Germination issues in the cultivation of *Echinacea angustifolia* in Tasmania

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Declarations

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge, contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

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Abstract

Echinacea angustifolia is a sought after medicinal herb originating from the Prairies of North America, however efforts to grow the species commercially have been hampered by seed dormancy. Seed pre-treatments had been successful *in vitro* but translation to field situations had been met with varying degrees of success. Pretreatment of seeds also carried a risk of loss because of reduced viability over the time involved with pretreatment.

This study investigated the most commonly used seed pretreatments and their effects on germination of *E. angustifolia* under laboratory, glasshouse and field conditions. It also investigated whether the manipulation of temperature and time would reduce the pretreatment period. The field experiment examined variation in sowing times and hence the use of natural climatic conditions to eliminate the need for chemical, or other, pretreatments.

Stratification or moist chilling is the most widely used method of seed pretreatment for overcoming dormancy in *E. angustifolia*. Six weeks of stratification in water at 4 ^{o}C increased the germination percentage of a dormant seedlot from 34% to 72%. A further improvement to 82% was obtained with stratification for two weeks in 10^{-3} M ethephon (2-chloroethylphosphonic acid). These pretreatments were also effective in a seedlot with low primary dormancy improving germination from 73% to 95%. In other experiments, ethephon was effective after only six to seventy-two hours at temperatures ranging from 4 ^{o}C to 25 ^{o}C. This suggested that seeds can be soaked in 10^{-3} M ethephon at ambient temperature for six hours on the day of sowing, therefore eliminating stratification for longer periods with associated risks. Ethephon pretreated plants,

however, appeared to have slightly reduced growth under glasshouse conditions.

The effectiveness of seed pretreatment was countered by other conditions in both the glasshouse and in the field. Seeds in a glasshouse trial appeared to have entered to a secondary dormancy state with 14-50% less germination than the laboratory results. These results were reflected in the field which had 33-51% less emergence. Temperature and water potential were the most likely causes of secondary dormancy. Results from experiments indicated that at 25 ^{o}C, germination and viability of seeds were affected when the water potential was -0.75 MPa or higher. This has implications in irrigation management during the early stages after spring sowings.

The field experiments demonstrated the effectiveness of dormancy loss through natural field conditions of untreated seeds for the winter month of July (southern hemisphere) when there were three months of soil temperatures below 10 ^{o}C. However, spring sowings required seed pretreatments to achieve reasonable field emergence.

The results are discussed in terms of practical management of this species as a commercial crop in Tasmania.

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Introduction

With the rapidly expanding use of medicinal herbs world-wide, Tasmania recognized the opportunity to become an international supplier of many medical herbs. *Echinacea angustifolia* was identified as a potentially high-value broad acre crop for Tasmania in the late 1990's. In 2002 interest surrounding the species had grown to the extent that it was listed as one of the ten most popular herbs (Wohlmuth *et al*., 2002**,** *cited in* the Australian Herbal Medicines Industry Feasibility Study, 2005).

Botanical Resources Australia Pty Ltd (BRA) is the world's largest pyrethrum producer and identified medicinal crops as an increasingly important part of their new business developments. BRA aimed to supply Echinacea to the herbal medicine industry in Australia and had committed to investment of research time and infrastructure. This PhD study was commissioned to focus on the field establishment of Echinacea under Tasmanian conditions.

For the present study, Chapter 1 reviews the literature and provides background information as to the cultural issues associated with Echinacea production, including seed germination and dormancy. Chapter 2 provides a description of the seeds used and the general materials and methods common to all experiments. Chapter 3 investigates the issue of seedlot variability. Chapters 4, 5 and 6 examine the issues of primary and secondary dormancy. Chapter 7 considers the findings of the individual experiments and combines the results to better understand the effect on seed germination and resulting establishment in the field.

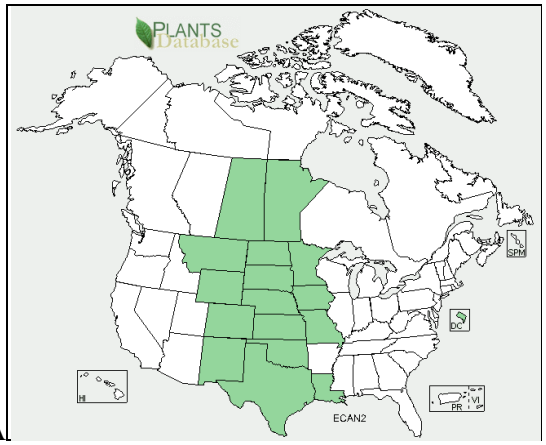
Early in the development phase of the Echinacea industry, difficulty in crop establishment of *E. angustifolia* became obvious, thought to be largely due to seed dormancy and seed germination issues. Therefore the main objectives of this thesis were to determine the optimum time for field sowing in Tasmania as well as investigate if the choice of sowing time can be used as a substitute for seed pre-treatment.

Chapter 1: Literature Review

1.1: Introduction and Background

Echinacea angustifolia DC (Asteracea: Heliantheae) is one of nine identified species of the genus *Echinacea* which are endemic to the North American continent, and one of three species (*E. angustifolia*, *E. purpurea* and *E. pallida*) that are commercially cultivated (Foster, 1991). The natural habitat of the genus is found in the Atlantic drainage system of the United States and extending into south central Canada east of the Rocky Mountains (McGregor, 1968). *Echinacea angustifolia* grows in the dry upland, often rocky parts of the prairies. There are two recognised varieties, *E. angustifolia* DC var. a*ngustifolia*, which ranges from south central Texas through the Great Plains north to Saskatchewan, Canada; and *E. angustifolia* DC var. *strigosa* which has a much smaller distribution from south central Kansas through central Oklahoma and north-eastern Texas (Fig.1.1A&B) (McKeown, 1999).

The use of Echinacea, especially *E. angustifolia* and *E. purpurea*, as a complementary alternative medicine or herbal supplement has gained popularity in recent years. It had been used extensively by indigenous Americans traditionally as a treatment for a wide variety of ailments from snakebites to toothache and rabies (Foster, 1991; Kindscher 1989).

A 

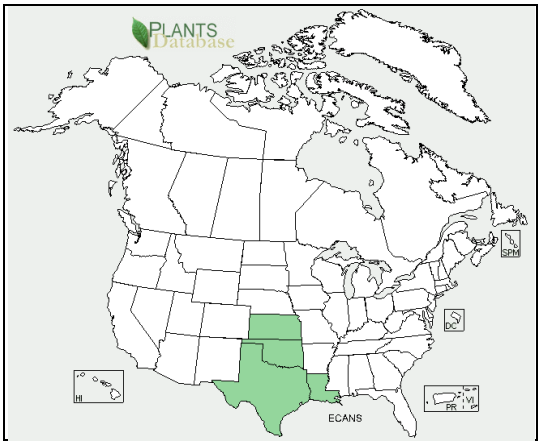
B 

Figure 1.1.1: Distribution maps for *Echinacea angustifolia* DC var. *angustifolia* (A) and *Echinacea angustifolia* DC var. *strigosa* (B) The maps do not distinguish between native and naturalised populations. [Plants Database, USDA].

1.2: Botanical description and taxonomy

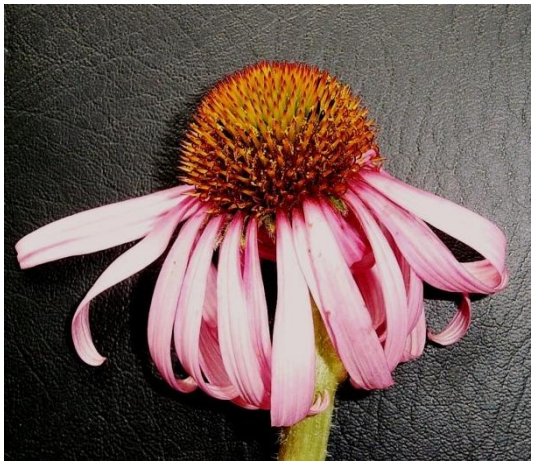
Echinacea angustifolia (Asteraceae: Heliantheae), commonly known as the narrow-leaf purple coneflower, is described as a herbaceous, perennial prairie forb growing to 30-60cm high, which thrives in mixed and tall grass habitats (Foster,1991; McKeown, 1999). The plant has a compact growth habit with an extensive branched tap-root system. First season's growth starts as a basal rosette of petiolate leaves with a central stem and subsequent growth occurs from the side of the crown.

The hispid, hirsute to strigose pubescent stems may be single or branched. The linear to lanceolate, 5-30cm long, rough hairy leaves are alternate with three to five veins running down the length of the blade. The inflorescence is borne singly at the end of the stem and has ray and disc florets (Fig.1.2.1) attached to a conical, spherical or sometimes flattened receptacle. Inflorescences appear in early summer to early autumn and the flowers open sequentially from the base of cone. Ray florets are sterile and have reflexed ligules, 2-4cm long, straplike with two or three notches at the end, and are generally white to pink or light purple in colour. Disc florets are 5-lobed and subtended abaxially by sharply tipped bracts, the paleas, protruding beyond the corolla (McKeown 1999). Pollen grains are generally yellow in colour (Fig.1.2.1a).

The species is insect-pollinated with 70-75% of visits by halictid bees (Wagenius & Lyons, 2010). Reproductive studies investigated the structure of the disk florets in relation to insect pollination (Wist and Davis, 2008), and style persistence, pollen limitation and seed set (Wagenius, 2004). Mature seed-heads are generally round, black and spiny (Fig 1.2.2), hence the name Echinacea from Greek „echinos' for hedgehog (Kindscher, 1989). *Echinacea* species appeared to hybridise readily (Fig 1.2.3b).

a.

b.



c.

d.

Figure 1.2.1: Whole flower head of *E. angustifolia* showing sequential maturation of disc florets with pollen grains (a &b), longitudinal section showing positions of florets and spiny bracts on the receptacle(c) and disc florets at different maturity (mature from base upwards) and ray floret (far right) with ligule (d). [Magnification: (a) x2.5, (b) & (c) x0.75, (d) x2].

Figure 1.2.2: Mature seed head of *Echinacea angustifolia* [Magnification 1.25].

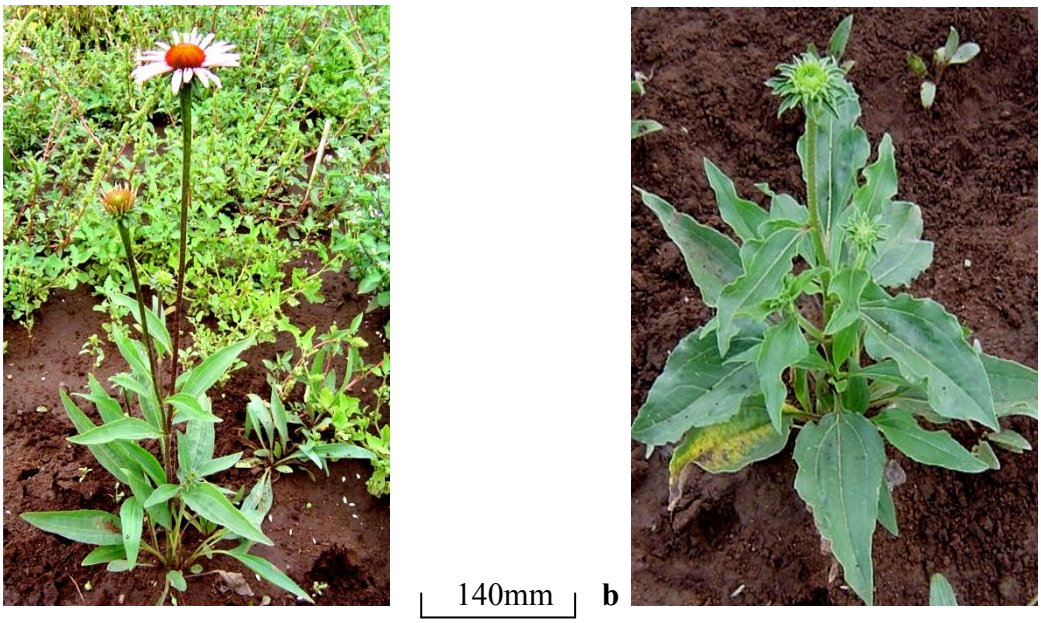


Figure 1.2.3: Two forms of *E angustifolia* appearing in a field trial (2006) at Glenora, Tasmania, Australia. The broader leaves (b) may have been due to hybridization with *E purpurea.*

The propagation unit is an achene or technically, a cypsela (Raven, Evert and Eichhorn, 2005) which is a fruit, although usually referred to as a seed (Fig. 1.2.4a-d). The endosperm is reduced to a light-coloured layer surrounding the embryo. Over this is a brown-pigmented testa, followed by a melanine-bordered layer of bast cells. The fruit

wall has several protrusions/ribs where channels (Fig. 1.2.5a&b) are located (Schulthess *et al*., 1991). Propagation can also be from crown divisions or root sections.

Figure 1.2.4: *E. angustifolia* achene: layers of fruit wall and testa removed to reveal an embryo covered by an endosperm. (a) whole achene, (b) achene with pericarp removed, (c) embryo with intact endosperm and (d) embryo with endosperm removed [Magnification: x10 (a&b); x12(c) and x12(d)].

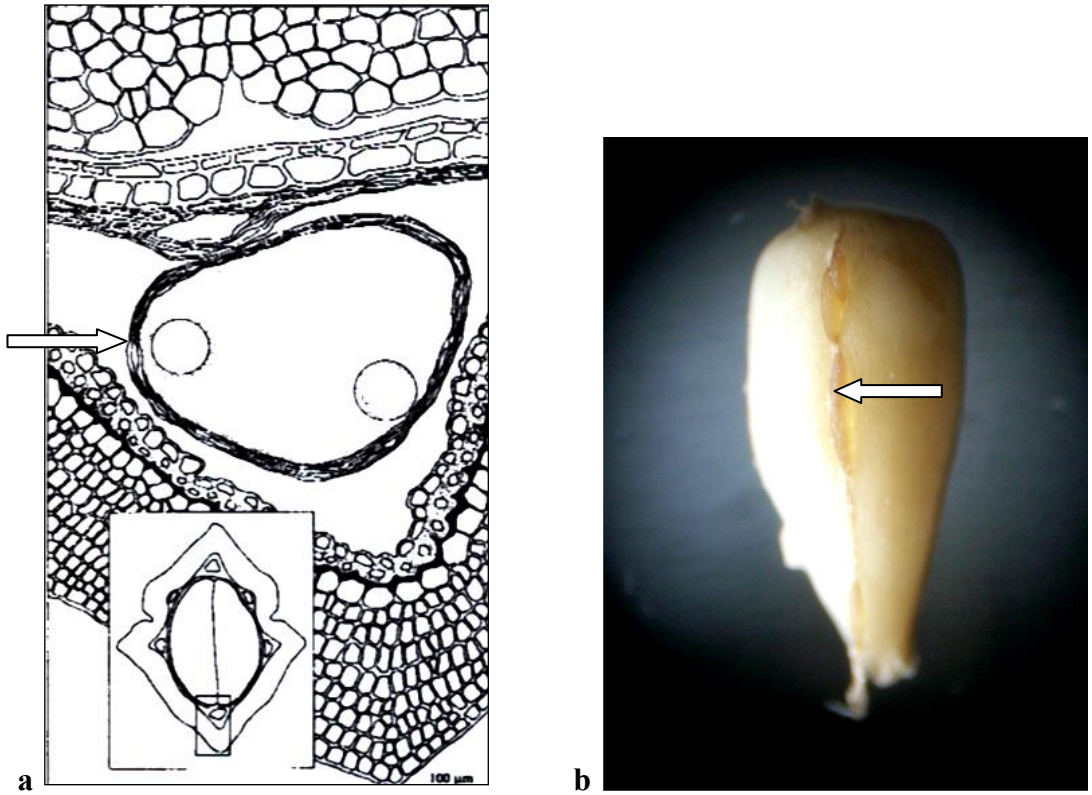


Figure 1.2.5: Cross-sectional diagram through an achene of *E. purpurea* (very similar to *E. angustifolia*) (Schulthess, *et al*., 1991) showing cell layers and arrow showing position of channels (a) and photograph of *E. angustifolia* seed showing position of a channel (b) [Magnification: x25].

Since 1743, *Echinacea* has been described as belonging to the genera *Rudbeckia*, *Brauneria* and *Echinacea* at different times. McGregor (1968) is credited with making a significant contribution to the taxonomy of the genus naming nine species and four varieties using morphological traits and chromosomal numbers. Previous use of ligule colour as a diagnostic characteristic is disputed by McKeown (1999) who described ligule colour as being developmentally dependent as well as geographical. Hybridization further complicates the problem of morphological variations (Mechanda *et al*., 2004). Binns *et al*., (2002) revised the taxonomy with a morphometric analysis on *Echinacea* and recognised two subgenera and four species with eight varieties as shown below:

Subgenera	Species	Varieties
Echinacea subg. *Echinacea*	*E. purpurea*	
Echinacea subg. *Pallida*	*E. atrorubens*	*E. atrorubens* var. *atrorubens*
	E. laevigata	*E. atrorubens* var. *neglect*
	E. pallida	*E. atrorubens* var. *paradoxa*
		E. pallida var. *angustifolia*
		E. pallida var. *pallid*
		E. pallida var. *sanguine*
		E. pallida var. *simulate*
		E. pallida var. *tennesseensis*

Therefore according to Binns *et al*., (2002), *E. angustifolia*, which has been normally considered a species on its own, is now a variety of *E. pallida*. Various phylogenetic (Mechanda *et al*., 2004; Flagel *et al*., 2008) and chemotaxonomic (Baum *et al*., 2001), metabolic profiling (Wu *et al.*, 2009) studies are available but the information presented is somewhat contradictory.

Because of the uncertainty of the taxonomic literature and also mainstream use of the name, *E. angustifolia* will be used as an accepted species name for the purpose of this work.

1.3: Industry background

The growth of the Echinacea industry, as in other medicinal herb cultivation industries, is governed by factors such as competition, demand, quality, access to plant or seed stock and communication and collaboration between the stakeholders. In Australia in the 1990's, Echinacea was emerging as a potentially high value crop and prices for *E. angustifolia* root were estimated to range from AUD$42-$100 per kg. The Australian market was not large but was growing at approximately 25% per annum with mainly imported *E. angustifolia* material (Walker, 1997). The potential marketability of this herb and the perception that it has a higher medicinal value attracted funding in Australia for projects, ranging from feasibility studies to agronomic and extraction methods, from Rural Industries Research and Development Corporation (RIRDC) and others. Echinacea was listed as one of the ten most popular herbs in 2002 (Wohlmuth *et al*., 2002**,** *cited in* the Australian Herbal Medicines Industry Feasibility Study, 2005). Australia's „green, clean' image has been considered an advantage in the global market especially so in the perceived better quality of organic medicinal herbs.

To date, success in the production of raw material has been hampered by several factors. The fragmented nature of the Australian industry with small isolated production areas, some with added demands associated with organic production, has been a disadvantage (Walker, 1997). The lack of agronomic information was also problematic with a dependency on web-based information mostly from the USA/Canada agri-websites

which were generally specific to their localities. *Echinacea angustifolia* seed prices were another factor in the slow development of the industry. Recent (January 2010) prices (Prairie Moon Nursery, USA) were US$150 per lb (AU$364.44 per kg) compared to US$30 per lb for *E. purpurea;* or 437 Euro (~AU$661) per kg (B&T World Seeds, 2009); and was even higher in Australia, AU$30/5g (Pleasance Herbs Seeds, NSW).

The cost of seed, together with inconsistent emergence due to dormancy issues, species integrity due to hybridization and the long (generally three years to harvest) period of growth, have contributed to the development of a contract-based industry. This was also more advantageous to the processor who had more control over the quality of the end product. Price competition was another factor in the industry's slow growth. In the case of *E. angustifolia*, where cultivation can be labour intensive, international competition was very strong where cheaper labour was readily available. More mature markets in the USA, Canada and Germany were also more able to withstand market vagaries (Yap, 2006).

The medicinal herb industry appeared to follow a trend of popular or fashionable belief whereby a specific herb would be in demand when there was positive media attention focused on it, such as perceived benefits relating to diseases/conditions. The market demand was therefore mainly driven by „casual' users rather than the „core' users who believe and rely on traditional herbal treatments (Yap, 2006).

According to Small (2004), new crops tend to follow a characteristic „cycle of profitability' (Fig 1.3.1), where Phase 1 is the research and development (R&D) stage, Phase 2 is the market expansion to saturation, Phase 3 is a stable period of profitability and Phase 4 is the decline due to factors such as market saturation, competition from

new products or generation of surpluses. Small (2004) suggested that Echinacea in Canada was at the end of Phase 3 in 2004, and any new investment was therefore inadvisable although those already growing the crop can still make a profit. This may also be applicable to the industry in Australia. „Stand alone' Echinacea products, including *E. angustifolia*, which were available on supermarket shelves and health food stores at the beginning of this work (2004) are now (2010) being replaced by combination products such as with *Allium sativum* (garlic), *Petroselinum crispum* (parsley), *Achillea millefolium* (yarrow), *Astragalus membranaceus* (milk vetch), *Hydrastis canadensis* (golden seal) and *Rosa canina* (dog rose) and compounds such as calcium ascorbate (vitamin C). *E. angustifolia* is hardly represented where Echinacea is named as an ingredient with *E. purpurea* being more commonly used.

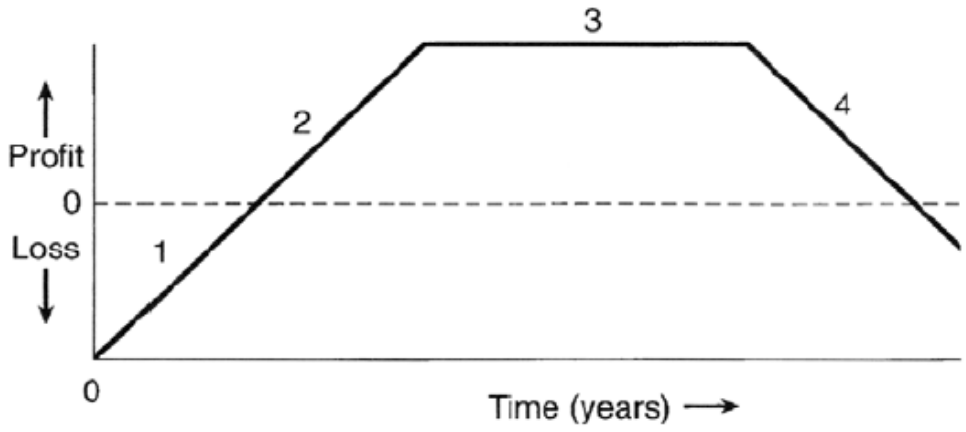


Figure 1.3.1: Cycle of profitability of new crops (Small, 2004) - R&D (1); market expansion (2); period of profitability (3); decline (4).

Although the popularity trend appears to be in decline, the opportunity still exists for farmers with established plantings to concentrate on seed production to take advantage of the current high seed prices.

In Australia, complementary medicines like *E. angustifolia* are regulated by the Therapeutics Goods Administration (TGA) under the Therapeutics Goods Act 1989. The TGA, with industry consultation, has developed Australian Regulatory Guidelines for Complementary Medicines (ARGCM) to assist sponsors of complementary medicines in meeting their legislative obligations (TGA, Dept of Health and Ageing). There are also standards and guidelines governing the production, harvesting and processing of the raw materials under the Australia New Zealand Food Standards Code. Whether government agencies routinely test for contents or quality is not clear, but manufacturers generally have trade standards for the raw materials, often to achieve a competitive advantage (Dowell, 2003).

1.4: Research & Development

There is a large collection of research on *E. angustifolia* (Parker & Parker, 2004) but the main focus has been on its medicinal efficacy. Recorded studies on *Echinacea* go as far back as 1897 with the isolation of an alkaloid by Lloyd (cited by Li, 1998), and in 1915 by Heyl and Hart (cited by Kindscher, 1989). Some early research (1947-1985) investigated uses other than medicinal, such as the isolation of echinolone as an insecticide (Jacobson, 1954, 1967).

Until fairly recently, research on *Echinacea* was mainly conducted in Germany. Bauer (1998), alone and in collaboration with other researchers (Bauer *et al*., 1988; Pietta *et al.*, 1998; Bauer and Remiger, 1989 and others), employed thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and micellar electrokinetic chromatography (MEKC) as analytical tools to characterise and differentiate among the different species. Alkamides (mainly isobutylamides), caffeic acid glycosides

(particularly echinacoside), polysaccharides, polyacetylenes have been found to be present in aerial parts and/or roots of *E. angustifolia*. Cynarin (another caffeic acid derivative) is characteristic of the species (Barnes *et al*., 2005; Woelkart *et al*., 2005; Bauer *et al.*, 1989). Binns *et al*., (2002) reported twenty-eight phytochemicals from *E. angustifolia* (measured by HPLC separation) but found distinct chemotypes within the species according to their natural geographic areas. This has complicated the issue of standardising commercial *E. angustifolia* preparations. The large variation in the quality of preparations has also brought about research to better differentiate and characterise between the species to enable standardisation and prevent adulteration by manufacturers (Kapteyn and Simon, 2002; Rogers *et al*., 1998; Schulthess *et al*., 1991; Widrlechner, & McKeown, 2002; Wolf *et al*., 1999).

Echinacea research is mainly directed at the relationship of its phytochemicals and the biochemical effects in humans. Studies on the efficacy of Echinacea as an anti-viral, anti-microbial, anti-inflammatory and immuno-stimulatory agent have created controversy with a number of randomised clinical trials with contradictory, inconsistent or inconclusive results. Turner *et al*., (2005) in their National Centre for Complementary and Alternative Medicine (NCCAM) and Office of Dietary Supplements (ODS) sponsored project, reported that extracts of *E. angustifolia* root, alone or in combination, "did not have clinically significant effects on infection with a rhinovirus or on the clinical illness that results from it". Similarly, Barrett *et al*., (2002) and Taylor *et al*., (2003) did not find detectable benefits for the common cold and upper respiratory tract infections in children respectively. Some of the studies, however, used subclinical doses, different parts of the plant or unassayed material which may have biased the conclusions. Other trials suggested that early intervention with Echinacea may result in reduced severity of symptoms in subjects with naturally acquired upper

respiratory tract infection (Goel *et al*., 2004). Shah *et al*., (2007), in their meta-analysis of several published trials, concluded that there was a benefit in decreasing severity and incidence of the common cold. There was also a suggestion that alkamides from the roots of *E. angustifolia* have immuno-modulatory effects (Woelkart and Bauer, 2007; Woelkart *et al*., 2005).

Although the phytochemical characterisation is comprehensive, the active components are still under investigation. A common issue for many of the published trials had been the difficulty associated with standardising preparations or obtaining standard commercial products. Sampson (2005) in the New England Journal of Medicine suggested that the mass of information available did not have sufficient heuristics or standards to establish clear efficacy of Echinacea as an alternative medicine.

Reports (Mullins, 1998; Mullins and Heddle, 2002) on allergic or adverse reactions such as anaphylaxis, rashes, delayed asthmatic reactions, muscle aches and pains and stomach upset, interactions with other medications (Izzo and Ernst, 2001) or medical conditions also precipitated more research into those areas. In the Australian Adverse Drug Reactions Bulletin (1999), the Adverse Drug Reactions Advisory Committee (ADRAC) was concerned that these reports suggested a predisposition to developing allergic reactions to *Echinacea* in individuals with asthma and hayfever.

Another area of research has been on the environmental impact that wild harvesting has had on *Echinacea*. Habitat fragmentation studies by Wagenius *et al*., (2007) and pollination, seed production and genetic diversity studies (Wagenius, 2004, 2010) have identify the need to preserve the wild population. Threats of decline of the wild populations of Echinacea in the United States of America and Canada, and the high

economic value of the product have accelerated the need to cultivate these species. This has necessitated research works on the agronomy of *Echinacea*, but to date however, studies have been limited. Of the nine species, three (*E. purpurea*, *E. angustifolia*, and *E. pallida*), are commercially traded as medicinal plants (Foster, 1991).

There is a general consensus on websites and technical advisories from agricultural sources, that the establishment of *E. angustifolia* and *E. pallida* are culturally more demanding with seed dormancy issues. *E. purpurea* is therefore the more widely cultivated of the three species. Most of the published reports addressing seed dormancy problems associated with the genus (Baskin *et al*., 1992; Sari *et al*., 2001; Feghahati & Reese, 1994) were laboratory-based experiments rather than investigations into the agronomic challenges. Experimental work, aimed at addressing dormancy issues are limited and mainly focussed on various seed pretreatments, environmental conditions, seed source and quality (Pill and Haynes, 1996; Sari *et al*., 2001, Macchia *et al*., 2001; Feghahati and Reese, 1994; Wartidiningsih and Geneve, 1994; Wartidiningsih *et al*., 1994; Parmenter *et al*., 1996; Samfield *et al*., 1991). These works have indicated the efficacy of cold stratification in breaking dormancy. The use of growth regulators such as ethephon (2-chloroethylphosphonic acid) has also been identified as an efficient means of shortening the required stratification time. However, it is unclear from these studies whether laboratory results were successfully translated into field situations.

Field management of *Echinacea* plants has received limited attention. There have been a few reports of research into diseases and pests. Letchamo *et al*., (2002) noted cucumber mosaic virus, broad bean wilt, and mosaic diseases, a shoot fungus (*Cercospora* sp.), and a root rot (*Phymatotrichum omnivorum*) on *E. purpurea* and *E. angustifolia*. Aster yellows caused by phytoplasma and transmitted by leafhoppers has

also been recorded as a significant disease in Alberta, Canada (Chang *et al*., 1999; Dyck, 2000). An introduced leaf smut, *Entyloma echinaceae* sp. *nov*., was found to cause severe damage to a crop of *E. angustifolia* in NZ (Vanky and McKenzie, 2002). Other common diseases such as Sc*lerotinia* rot, *Pythium* and *Rhizoctinia* in seedlings were also noted (Chang *et al*., 1998). Pests such as Lygus bug, aphids, thrips, grasshoppers and stinkbugs can also be a problem (Manitoba Agriculture and Food, 2001). Letchamo *et al*., (2002) reported sunflower moth (*Homoesoma electellum*) as a significant pest with young larvae destroying flowerheads and older larvae tunnelling through stems causing secondary infections and opportunistic diseases.

1.5: Cultural issues

The cultural issues associated with *E. angustifolia* are related to its natural habitat. As a native of the North American prairie (considered an arid to semi-arid region), *E. angustifolia* is drought and frost-tolerant, and is adapted to a range of 250-750 mm of precipitation annually, with temperatures which can range from -40 ºC in the winter to more than 40 ºC in the summer. As a dry, rocky upland species, *E. angustifolia* is subjected to high irradiation in summer and high evaporation from wind (Risser *et al*., 1981; Hayden, 1998). It therefore prefers sunny conditions and free-draining soils with a pH range of 6-8 (Czech, 2002).

In the wild, the life cycle *E. angustifolia* is timed to allow for the natural release of dormancy. The plants flower in summer and flowering lasts for about a month. Seeds start to mature in autumn and continue over a period of months dispersing throughout the winter and even into the early spring, allowing the seeds to undergo a natural stratification process (Czech, 2002). Seeds can remain dormant for more than a year

when conditions are not conducive for growth (Kindscher, 2006) and may require an extended period of natural stratification (Baskin and Baskin, 1993). Seedlings germinate in spring and may only have one true leaf for the first growing season and may require up to three years to form the basal rosette from which the flower stalk appear (Weaver and Fitzpatrick, 1934 cited in Kindscher, 2006), whereas mature plants start as soon as the danger of frost is over (Kindscher, 2006). *E. angustifolia* is adapted for survival in the grasslands with the leaves from the rosette pushing back as they grow. The long tap roots (1.6-2.4 m) occupy a different soil horizon from grasses and are able to resprout after a grass fire (Czech, 2002). Prairie fires are beneficial to *E. angustifolia* by removing trees and shrubby growth (Kindscher, 2006). Wild *E. angustifolia* is reportedly able to survive for between eighteen and forty-four years (Hurlburt, 1999).

Most technical advisory literature has suggested a stratification period for the seeds before sowing, but the New Zealand Institute for Crop & Food Research (Douglas and Parmenter, 2001) found that seeds sourced from Europe had 80-90% germination without chilling treatment. Although direct seeding had advantages of being less labour intensive than transplanting, unreliable emergence is presently an unresolved problem. Smith-Jochum and Albretcht (1987, 1988) found direct-seeded *Echinacea* plants were shorter, had fewer flowers and reduced root weight. Weed competition is also a bigger problem with direct seeding (Little, 1999). With the high costs of seeds, transplanting is an option. Transplanted *E. angustifolia* established well in a trial in Tasmania, but there was a lack of persistence with no second year re-emergence (Buntain, 1999). The author stressed site selection as being critical for the *Echinacea* species to survive soil saturation over winter.

Row spacings of 0.4m with plant spacing of 0.3m (plant density of 16 plants/m^2) is usual but an experimental trial in NZ (Douglas and Parmenter, 2001) found plant density of 25 plants/m^2 gave the highest yield for *E. purpurea*. Raised beds are advised for *E. angustifolia* as it requires very good drainage. A friable soil with low clay content is important for ease of root harvest and cleaning.

Very good weed control is one of the most frequently mentioned factors as establishment of the plants is slow initially. In the NZ trial, *E. purpurea* tolerated pendimethalin, oryzalin and a combination of oryzalin and chlorpropham at planting, and terbacil, diuron or chlorpropham once established (Douglas and Parmenter, 2001). Gesagard® 500SC (triazine) at one litre per hectare (depending on soil type) will control most dicotyledon weeds and Fusilade® Forte (carboxylic acid derivative) at 2.5 L per hectare will control grasses (Groom, *pers comm*., 2005). However, the effects of herbicides on the plant itself, as well as effects on the marketable components are not documented. Weed control in organic production can be as high as $8,000-$10,000 per ha per annum (Walker, 1997). Buntain (1999) also identified weed management as the greatest expense in time and cost in a Tasmanian field trial.

As the biggest industry issue is the emergence of seedlings and the establishment of plants, the next section will review literature on seed germination.

1.6: Seed germination

Bewley and Black (1994) defined germination as a process which incorporates a series of events that "begins with the uptake of water (imbibition) by the (quiescent dry) seed and ends with the start of elongation by the embryonic axis, usually the radicle".

As described by Bewley (1997), seed imbibition is triphasic (Fig.1.6.1) with Phase I being a rapid uptake of water, followed by Phase II which is a plateau phase when water uptake slows down. The seed water content rises only very gradually or not at all during phase II (Bradford, 1995). Phase III is another period of rapid intake, but only occurs if the germination process is complete and there is radicle emergence and continued growth. Imbibition is governed by the water potential which is the sum of the osmotic, matrix and the pressure potentials (Bewley and Black, 1994).

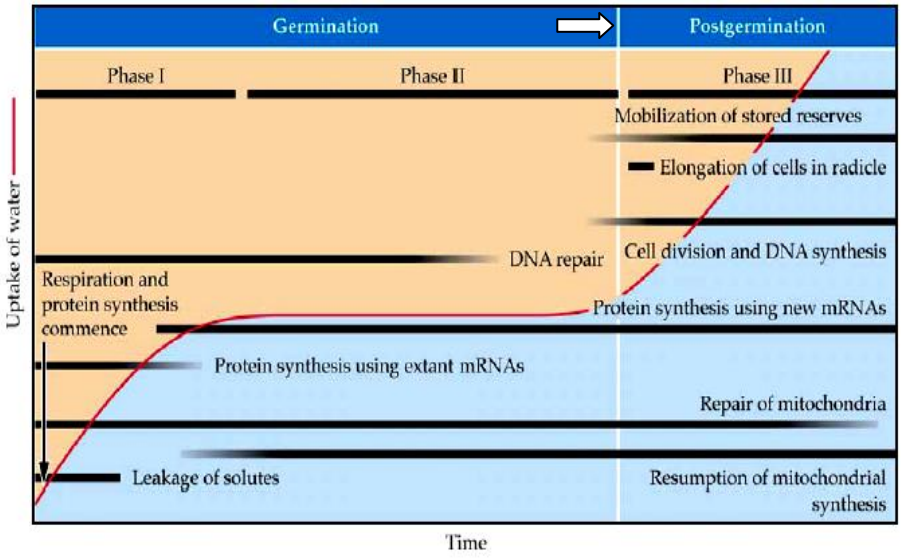


Figure 1.6.1: Seed germination: Triphasic water uptake. „Time course of major events occurring during germination and subsequent post-germinative growth' (Bewley, 1997). Arrow marks the completion of germination.

It is generally accepted that glycolysis, the oxidative pentose pathways and the citric acid cycle resume during phase I, and there is a big increase in oxygen consumption. Protein synthesis, respiration, enzyme activities, subcellular structural changes, cell elongation, mitochondrial development and activities take place as the seed is hydrated. The anatomic, genetic, metabolic and hormonal events are discussed extensively in Bewley and Black (1983, 1994), Kigel and Galili (1995) and Khan *et al*., (1978).

Hormonal control of germination has been discussed by Schopfer and Placy, (1985), Groot and Karssen, (1987), Karssen, *et al.*, (1989) and more recently by Kucera *et al*., (2005). It is generally accepted that gibberillins (GAs) promote germination, and abscisic acid (ABA) has a role in inhibition of germination. Hormonal influences do not affect the seeds solely at germination, but also play a role during seed development and maturation (Karssen *et al*., 1983; Karssen and Lacka, 1986, Ali-Rachedi *et al*., 2004). ABA is important during seed development in the induction of dormancy and desiccation tolerance. GA does not seem to have a major role in seed development. However, GAs, endogenous or exogenous, are essential for germination. Studies with GA-deficient mutants have shown that exogenous GA is essential for germination in tomatoes (Groot and Karssen, 1987) and in *Arabidopsis* (Karssen, 1995).

Phytochrome-control in germination has been studied since 1920 (Garner and Allard) and has been regularly investigated by various researchers (Taylorson and Hendricks, 1968; Romero *et al*., 2005; Finch-Savage and Leubner-Metzger, 2006). Evidence seems to suggest the phytochrome changes are controlled or associated with hormonal activity which is temperature-dependent (Thomas, 1992). Temperature also has a role in phytochrome-related germination responses (Taylorson and Hendricks, 1968; Van der Woude and Toole, 1980).

In addition to internal mechanisms, there are external factors influencing germination. Environmental conditions must be favourable for germination to proceed. The effects of temperature (Coolbear, *et al*., 1991; Probert, 2000; Whithead and Sutcliffe, 1995; Corvell *et al.*, 1986; Larsen, *et al*., 2004) and light (Vidaver, 1977; Inoue and Nagashima, 1991; Thomas, 1992; Pons, 2000; Walck, *et al.,*. 2000; Steckel, *et al*., 2004) have been well researched. Well documented too, are studies on the chemical

environment (Hilhorst and Karssen, 2000; Batak, *et al*., 2002) and gaseous environment (Corbineau and Côme, 1995; Jones and Hall, 1981). These external factors usually interact with internal controls such as hormones and phytochrome receptors. For example, Roth-Bejerano *et al*., (1999) found that light induces germination of photoblastic „Ritsa' lettuce seeds by inhibiting ABA synthesis and decreasing ABA sensitivity.

Despite both environmental and internal control mechanisms for seed germination, some *E. angustifolia* seeds still fail to germinate. It is in these instances that seed dormancy may be attributed to the inability to germinate.

1.7: Seed dormancy

Seedlots of *E. angustifolia* described by Baskin *et al*., (1992, 1998) and Feghahati and Reese (1994) had a germination percentage below 40, that is, less than forty percent of the seeds completed the germination process. The rest of the seeds were considered to be dormant. Seed dormancy and germination are concurrent processes (Eira and Caldas, 2000) as the dormant seed can commence germination and achieve different stages without progressing onto phase III (see Fig. 1.6.1). Dormancy is an adaptive trait with a temporal and/or spatial function, which delays germination until environmental conditions are suitable for seedling growth. This allows a wider range of conditions and times (seasons) at which germination can occur. Seeds with inherent dormancy are referred to as having primary dormancy (Crocker and Barton, 1957; Villiers, 1972; Bewley and Black, 1982). This primary dormancy is initiated during the development/maturation of the seed.

Secondary or induced dormancy can develop in non-dormant seeds or in seeds that have been released from primary dormancy, if the seeds are held under conditions unfavourable for germination (Momoh *et al*., 2002; Gulden *et al*., 2004). Unsuitable temperature, moisture content, illumination or anoxia may induce secondary dormancy in a range of species (Bewley and Black, 1994). Temperature increases have a marked effect on the rate of induction of secondary dormancy in imbibed seeds (Murdoch and Ellis, 2000). There are no references in published literature regarding secondary dormancy in *E. angustifolia* but the erratic nature of emergence in the field may be suggestive of secondary dormancy.

Besides environmental conditions, some chemicals such as GA, nitrates, cyanides and ethephon have been known to alleviate seed dormancy. Ethephon as a dormancy-breaking agent for seeds has been widely tested and its dormancy-breaking effects have been demonstrated. Kepczynski *et al*., (1996) found it effective in dormant *Amaranthus retroflexus,* and similarly in *Manihot glaziovii* (Drennan and van Staden, 1992), *Triteleia laxa* (Han, 1993) and *Matricaria maritima* (Mekki and Leroux, 1991). Ethephon efficacy has also been demonstrated in *E. purpurea* (Li *et al*., 2007) and *E. angustifolia* by Feghahati and Reese (1994) and Macchia *et al*., (2001). Ethephon (2-Chloroethylphosphonic acid**:** $C_2H_6Cl_3P$) commonly known as Ethrel® or Florel®, is a plant growth regulator usually used in agriculture to promote pre-harvest ripening/synchrony/colour of fruits, tomatoes (Murray, 2000), beets, berry crops (Avenant and Avenant, 2005), and coffee (Winston *et al*., 1992). It is also used to promote flower initiation and breaking apical dominance in apples and other fruits, vegetables, nuts and trees. It is used as a crop terminator in cotton to facilitate harvesting (Reddy, 1995) and to induce premature flower or fruit drop as a means of crop regulation in apples and pears (Bound *et al*., 1991, 1993). It is also used to

stimulate latex flow in rubber trees, prevent lodging in cereals, maize, and flax. Atta-Aly *et al*., (1999) also found that a low level application at early fruiting stage can extend cell division, and therefore increased fruit size in tomatoes. The mode of action in the seeds is not fully understood, but ethylene released during and after exposure is generally accepted as the active component.

Cohn *et al*., (1989), found a dormancy-breaking response to weak acids which was highly dependent on pH, in addition to solution concentration and exposure time. However, Feghahati & Reese (1994) looked at phosphoric acid (at the same solution concentration as ethephon), and water acidified to pH 3.8 (same pH as ethephon) with hydrochloric acid, and found germination in each case was significantly less than in seed treated with ethephon at the same pH and concentration.

Ethephon increased adventitious root formation and root dry weight in mung beans (*Vigna radiate*) (Pan *et al*., 2002), and counteracted the effects of root restriction and increased lateral shoot development in Petunia (*Petunia x hybrid*) seedlings at the same root volumes (Haver and Schuch, 2001). There is no literature about the effects of ethephon on *E. angustifolia* seedlings and as *E. angustifolia* is grown for root production, the effects of ethephon on root development need to be considered. Also, given that ethephon has the other actions of promoting senescence and ripening, there are no known studies as to its possible toxic effects on seeds or negative effects on subsequent seedling growth and development.

Ethephon decomposes into ethylene, phosphate and chloride ions in aqueous solution above pH 5, but is considered stable at pH 4 and below (Gianfagna, 1995). Warner and Leopold (1969) determined by gas chromatography that the gas evolved is ethylene and

found increasing evolution with increasing pH. Although experimental use of ethephon began in the early 1970s, it is still unclear what other products are formed after ethephon enters a biological system. At pH 7, conversion to ethylene is about 80% (Domir and Foy, 1978) and has been shown to be temperature-dependent with half-lives of 100hr and 4hr at 15 and 35ºC respectively (Kreicberg *et al*., 1984). Kepczynski, Corbineau and Côme (1996) also found a correlation with temperature in *Amaranthus retroflexus* with almost nil ethylene release at 20ºC to nearly 100% at 35ºC. Sensitivity of ethylene release to temperature has also been an important issue in the commercial use of ethephon in apple crop regulation, (Bound *et al*., 1993). The solution is also sensitive to UV radiation (Ethrel-MSDS).

1.8: Ethylene in plants and seeds

With the assumption that ethylene is the factor in ethephon that governs the release from dormancy, it is appropriate to review its biological activities. Because of its importance in the post harvest management of horticultural crops, the literature on ethylene is voluminous.

Ethylene ($H_2C=CH_2$) is the only gaseous plant hormone mainly linked to senescence, induction of fruit, flower and leaf abscission, fruit maturation and seed germination (Bleecker and Kende, 2000; Yoo *et al*., 2009). Ethylene also appears to have a role in the determination of sex expression in flowers of monoecious plants e.g. in the *Cucurbitaceae* family (Abeles, 1973; Raven *et al*., 1992); induction of flowering in bromeliads; synchronous flower development of pineapples, promotion of root initiation, dwarfing (reduction of internodal elongation) and tuber and bulb formation

(Abeles, 1973). It is now known to be involved in growth and developmental processes including seed germination (Kepczynski and Kepczynska, 1997; Abeles, 1973).

Ethylene can essentially be synthesized by all parts of a plant – leaves, stems, flowers fruits, roots and seeds (Yang and Hoffman, 1984). Ethylene biosynthesis is a complex process and whilst it is likely that the general picture of biosynthesis and control mechanisms is common across different plant tissues, there is little published information on the biochemistry of ethylene in germinating seeds. The pathways of ethylene biosynthesis have been investigated by many researchers including Abeles (1973), Lieberman (1979), Yang and Hoffman (1984), McKeon *et al*., (1995) and Smith and Hall (1984). Methionine is the main precursor in ethylene biosynthesis (Yang and Hoffman, 1984). Lieberman and Mapson (1964) first found methionine greatly increased ethylene production while working on one of their model systems. This was supported by others working on different systems (cited in Yang and Hoffman, 1984). Further, Adams and Yang (1977) identified S-adenosylmethionine (SAM) as an intermediate, by feeding labelled methionine into apple tissue and showing 5'–methylthioadenosine (MTA) was formed together with ethylene. Adams and Yang (1979) continued their work with methionine metabolism in air and nitrogen and discovered that, in air, methionine is rapidly converted to ethylene, but in nitrogen, it is metabolised to 1-aminocyclopropane-1-carboxylic acid (ACC)**:**

Methionine ⟶ SAM ⟶ ACC ⟶ ethylene

From there, it was found that there is a recycling pathway whereby methionine is regenerated, called the methionine cycle (also known as the Yang cycle). The review by Yang and Hoffman (1984) stated that it is now accepted that ethylene is synthesized from methionine via SAM and ACC and that methionine is regenerated in the process. There are a number of enzymes involved in the biosynthesis and regulation of ethylene.

Methionine adenosyltransferase catalyses the conversion of methionine to SAM, and the process is ATP-driven. The ACC synthase then catalyses the conversion of SAM to ACC and thereby regulates the production of ethylene. The level of ACC synthase is affected by changes in hormones, the growth stage of the plant, the environment and other events. Preclimacteric fruits, for example, have a limited ability to covert SAM to ACC and also ACC to ethylene. Auxin induces synthesis of ACC synthase through RNA and protein synthesis, thus producing more ACC and resulting in higher levels of ethylene (Yang and Hoffman, 1984). ACC oxidase drives the conversion of ACC to ethylene (McKeon *et al*., 1995). The marked increase in ACC oxidase during senescence and ripening is due to an increase in transcription (Woodson *et al*., 1992). At the other end, where ethylene production is not desirable, N-malonylaminocyclopropane-1-carboxylic acid (MACC), driven by ACC N-malonyl transferase, serves as a sink that allows the level of endogenous ACC to be depleted, thus reducing ethylene synthesis (McKeon *et al*., 1995).

The biosynthesis of ethylene is believed to be regulated by several factors**:** hormonal or environmental, or inhibitors. Hormones such as auxin and ethylene itself can regulate ethylene production. Exogenous gibberellins have been found to have a small promotive effect on ethylene production in bean, blueberry flowers, orange fruit and leaves, potato tubers and peanut seeds (Abeles, 1973) but not in others. Some investigators found that cytokinins synergistically enhanced the ability of auxin to increase ethylene release and also extend the period of the auxin effect on seeds (Abeles, 1973). Cytokinins application results in greater ACC synthase activity and accumulation, whereas abscisic acid (ABA) may apparently inhibit induction of ACC synthase in drought-stress but may also promote ethylene production by stimulating ACC levels in some plant tissues (literature cited in McKeon *et al*., 1995). ACC was reported to accelerate germination

rate in *Echinacea* species (Wood, 2006). Ethylene itself is known to regulate ethylene biosynthesis either promoting or inhibiting the cycle. During autocatalysis, ethylene production is initially due to ACC conversion to ethylene, and later a big increase in ACC synthesis (McKeon *et al*., 1995). Auto-inhibition is known to occur in flavedo tissue of citrus fruit and it was suggested that it resulted from inhibition of ACC synthase activity (Riov and Yang, 1982).

Environmental stresses such as high temperature, chilling, flooding, drought, wounding, infections, result in the biosynthesis of stress ethylene which is most likely controlled by the conversion of SAM to ACC (Yang and Hoffman, 1984). Very high temperature (> 35ºC) causes ACC oxidase activity to drop and is lost at 40ºC (McKeon *et al*., 1995). In apples, the optimum temperature for ethylene production is 30^{o}C and ceases at 40^{o}C (Abeles, 1973). Ketring and Morgan (1969) reported a five-fold increase in the rate of ethylene production in the germination of dormant Virginia-type peanuts that were heat-shock (40-45^{o}C) treated. Ethylene levels determined prior to radicle emergence was ten times more at 33^{o}C than at 23^{o}C in non-dormant cocklebur seeds (Katoh and Esashi, 1975). Hall *et al*., (1987), however, advised caution when considering temperature and ethylene because the solubility of the gas changes with temperature, and concentrations applied in the gas phase may not correspond with concentrations in the liquid phase in the plant tissues. In dormant *Amaranthus retroflexus* seeds, high temperatures (35-40ºC) are required for germination in the dark, and ethephon treatment is very efficient only at 30ºC (Kepczynski, *et al*., 1996). The indication was that sensitivity of the seeds to ethylene decreases with decreasing temperatures. Burdett's (1972) work with Grand Rapids lettuce seeds suggested germination inhibited by high temperature pretreatment was associated with a reduction in the rate of ethylene production, and can be reversed

by chilling treatment. Freezing temperatures too, can cause an increase in ethylene level (Abeles, 1973).

Anaerobic conditions caused by water-logging can induce the synthesis of ethylene by the shoot, causing adventitious root growth, petiole epinasty and leaf senescence (Jackson and Campbell, 1976). Water-logging blocks the aerobic conversion of ACC to ethylene in the roots of tomato plants but causes increased synthesis of ACC which is transported to the shoots where it is converted to ethylene aerobically (Bradford and Yang, 1980). Trauma-induced stress ethylene had been utilised, although not recognised, a long time ago when figs were slashed to cause ripening. It was also the cause of harvest loss where bruised or damaged fruits can lead to rotting in whole storage containers. It is not clear if injury during imbibition of seeds (Bewley and Black, 1982) causes the production of stress ethylene which enhances germination of ethylene-requiring species.

Carbon dioxide (CO_2), generally considered to be an ethylene antagonist, is used in controlled atmosphere fruit storage where high levels delay ripening. It is believed to act by competing for ethylene-binding sites (McKeon *et al*., 1985). However, CO_2 also promotes production of ethylene as it is required for the conversion of ACC to ethylene by ACC oxidase activity and is an essential activator although the mechanism is not clear. Other work has suggested that CO_2 promotes ethylene evolution by inhibiting ethylene retention or breakdown (McKeon *et al*., 1995).

Like CO_2, light can promote or inhibit ethylene production. The effects of light and CO_2 are rapid and reversible (Bassi and Spencer, 1982). Light effects most likely act at the ACC to ethylene step. Production was inhibited in bean seedlings by a short exposure to

red light which can be reversed by far-red irradiation, suggesting phytochrome mediation (Vangronsveld *et al*., 1988; Beall *et al*., 1996, Foo *et al*., 2006). Other studies proposed that inhibition is related to the photosynthetic system (Yang and Hoffman, 1994). There is a relationship between light and CO_2 which is not clear, as in the presence of sufficient CO_2, light may also stimulate ethylene production in some green tissues (Grodzinski *et al*., 1982, 1983).

Other inhibitors of ethylene act at different steps of ethylene biosynthesis. Pyridoxal enzyme inhibitors were shown to inhibit ACC synthase (Rhando, 1974; Murr and Yang, 1975). Inorganic ions, Co^{2+}, Ni^{2+} and Ag^{+} may interfere with ethylene binding (Lau and Yang, 1976a). Ca^{2+} possibly delays senescence via preservation of membrane integrity. But Ca^{2+} has also been known to stimulate ethylene production synergistically with cytokinins (Lau and Yang, 1974) or Cu^{2+} in mungbean hypocotyls (Lau and Yang, 1976b).

Nord and Weicherz (1973) were credited with the first report of the effect of ethylene on seed germination in 1929. Since then, ethylene and germination had been the subject of many research studies. In seeds, both dormant and non-dormant types produce ethylene during germination. However, the level of ethylene in dormant seeds may be below the threshold required for the completion of germination (Kepczynski & Kepczynska, 1997) and therefore there is no radical emergence. Fu and Yang (1983) found that radical protrusion correlates with a peak in ethylene production. The effects of ethylene on seed germination appear to be species-dependent. Even within species, such as *Xanthium pensylvanicum* (Katoh and Esahi, 1975), the response can be different depending on the position of the seeds within the seed-pod. Ethylene is effective on dormant weed seeds such as *Amaranthus caudatus, Amaranthus retroflexus, Rumex*

cripus, Spergula arvensis, Chenopodium album and parasitic plants, *Oranbanche rumosa* and *Striga* species. For example, ethylene injected into soil to a depth of 30cm and horizontally to 70cm, can promote up to 90% germination of *Striga asiatica* (Corbineau and Côme, 1995).

Ethylene and seed germination has also been studied in several crop species. Toole *et al*., (1964) reported that dormant Virginia-type peanut seeds were induced to germinate by ethylene. Ketring and Morgan (1969) found that an extremely dormant variety of Virginia-type peanut seeds produced low levels of ethylene when imbibed, but after treatment with ethylene, the production from the embryonic axis was greatly increased. Similarly, Corbineau and Côme (1992) also found ethylene increases germination in sunflower seeds. Abeles (1986) found a relationship between dormancy in lettuce with ethylene. Esashi and Leopold (1969) reported that dormant varieties of *Trifolium subterraneum* produced less ethylene and that germination was preceded by an increase in the gas. There is some indication that response to ethylene is dependent on the time of application. Schőnbeck and Egley (1981) found that exogenous ethylene had the greatest effect in the first 20 hours of imbibition in redroot pigweed (*Amaranthus retroflexus*) germination.

Although ethylene is a well documented subject, it is still unclear whether its application stimulates the production of endogenous ethylene or the exogenous application itself is the cause of dormancy release. The biosynthesis of ethylene is well researched, but the physiological and biochemistry effects on dormancy release are still unclear.

1.9: Conclusion

The taxonomic revision of *E. angustifolia* is inconclusive at this stage and *E. angustifolia* will be considered as a recognised species in this work. The propagation unit is an achene but will be referred to as a seed here for simplicity.

Echinacea angustifolia is an endemic species of the upland, rocky parts of the North American prairies with climatic conditions of those regions having summers of low rainfall, high temperatures, irradiation and evaporation, and winters of very low temperatures and snow cover. The species has adapted to these conditions with very long, extensive tap roots, and foliage die-down in winter, and primary seed dormancy. Dormancy allows emergence over a range of temperatures to ensure survival of the species. As part of a mixed species in the prairie system, dormancy also allows emergence at a time when the other species are not in active growth.

The primary dormancy in *E. angustifolia* can be alleviated by pre-treatments such as cold stratification or ethephon. However, even with pre-treatments, there is still a problem with erratic emergence in the field. This may be due to induction of secondary dormancy. The following work will therefore focus on these two issues.

Chapter 2: General materials and methods

2.1: Introduction

This chapter describes the plant materials and methods common to the field, glasshouse and laboratory experiments in further chapters. Storage, handling, sampling and testing procedures are outlined. Germination tests are performed as per Statistical procedures used in this thesis are described. Where materials and methods differ, details will be provided in the relevant chapters.

2.2: Plant material

The three samples of *E. angustifolia* seeds were sourced from commercially grown plants from:

- 2002 harvest from a Botanical Resources Australia Pty. Ltd (BRA) experimental trial in Ulverstone, Tasmania
- 2003 harvest (light fraction) from Yaxley Farm, Ulverstone, Tasmania, provided by BRA
- Prairie Moon Nursery in Winona, MN, USA – harvested in 2004 in Grant Co. South Dakota stored at 60 ºF (15.5 ºC) before despatch.

Seeds were stored in plastic ziplock bags at 4 ^{o}C on arrival and for the duration of the experimental period. These seedlots will be referred to in the following chapters as SL2002, SL2003 and SL2004 respectively.

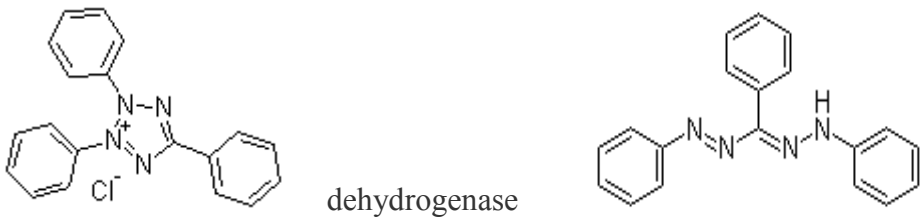
SL2003 was split into five density grades on a density gradient table (Westrup LA-K, Denmark) and seeds from the two highest densities (grades four and five) were

combined. These were then put through a South Dakota seed blower (Seedburo, Chicago, USA) to remove the lighter debris (settings were determined by visual inspection). The seeds were then hand-sorted to remove any trash/broken seeds that remained. SL2002 and SL2004 were supplied in small quantities (less than 85 g) and were hand-sorted only.

Seed samples for germination tests were selected using the method of repeated hand halving (ISTA, 1999). Separate samplings were done for each sub-sample representing a replicate, and fifty seeds were then counted using a Contador (Pfeuffer, Germany) seed counter from each sub-sample.

2.3: Seed viability test

The topographical biochemical, Tetrazolium (TZ) test (Cottrell, 1948; ISTA, 1999, Sawma *et al*., 2002), was used for quick estimation of the viability of the seed samples and also to determine the viability of ungerminated seeds at the end of a germination test. 2,3,5-triphenyltetrazolium chloride (TTC or TZ) acts as an indicator of viable dormant seeds. The TZ test is a dehydrogenase activity test whereby 1,3,5-triphenylformazan (TPH), a stable red non-diffusible substance, is produced in living cells by the hydrogenation of the colourless TZ (Fig. 2.3.1).



TTC — $C_{19}H_{15}N_4Cl$ → TPH — $C_{19}H_{16}N_4$

Figure 2.3.1: Reduction of 2,3,5-triphenyltetrazolium chloride to triphenylformazan (chemBlink, 2010)

2.3.1: Preparation of seed for staining

An aqueous solution of 1% TZ of pH 7 (±0.5) was prepared by dissolving TZ in a buffered solution of two parts KH_2PO_4 (9.078 g in 1000 mL water) and three parts of $Na_2HPO_4.2H_2O$ (11.876 g in 1000 mL water). The container was wrapped in double layers of aluminium foil to exclude light.

The ISTA International rules for seed testing (1999) do not include a specific method for *Echinacea* but they were used as a guide for this study. Four replicates of 50 seeds on two layers of 80 mm Advantec® filter paper in petri dishes were imbibed with 10 mL of deionised water for twelve hours at 20 ^{o}C. The distal end of the seed was transversely cut such that a very small part of the top of the cotyledon was removed (Fig.2.3.2). This ensured the embryo was freed from both the pericarp and endosperm when the seed was gently pressed with the flat of a scalpel blade. The embryos were kept moist until the whole replicate was processed, before placing onto filter papers in fresh petri dishes with 10mL of the 1% TZ solution. The petri dishes were then wrapped in foil to exclude light as TZ is reduced by light (Atkinson, Melvin and Fox, 1950, ISTA, 1999), and the embryos allowed to stain for 24 hrs at 25^{o}C.

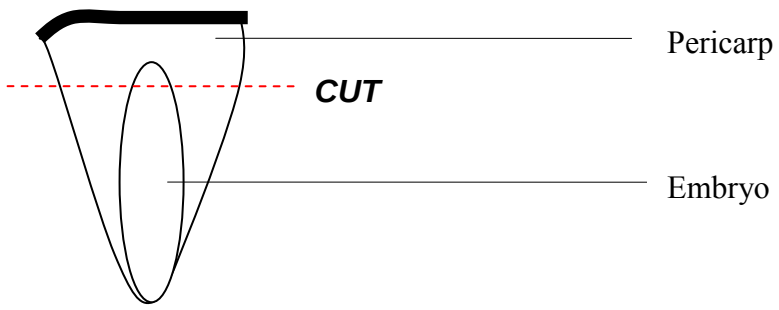


Figure 2.3.2: Diagrammatic representation of transverse cut through pericarp and cotyledon of *E. angustifolia* seed.

2.3.2: Evaluation

Cells were considered as living when they stained red and as non-living when they remained white or discoloured. Seeds stained red completely are rated as viable (Fig 2.3.2-2.3.4). Where partial staining occurred, colour differences and tissue soundness were also taken into consideration in assessing the seed viability (ISTA, 1999; Miller, 2005). According to Copeland (1995), the correct interpretation and evaluation of the stained seed are subjective and require a great deal of experience. The TZ tests in this study were therefore only used as a guide to seed viability and for comparison with germination tests.

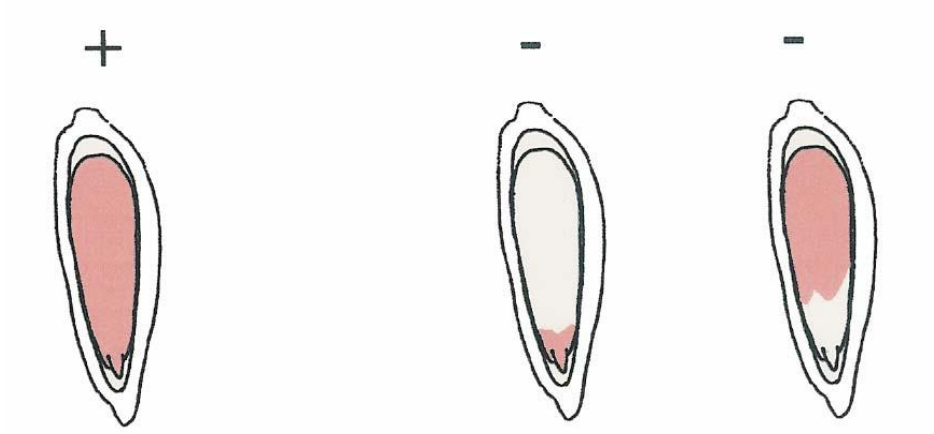


Figure 2.3.2: TZ evaluation of lettuce seeds with viable (a) and non- viable (b&c) (Meyr, 2006)

Figure 2.3.3: TZ results of 100 *E angustifolia* SL2002 seeds showing both viable and non-viable seeds

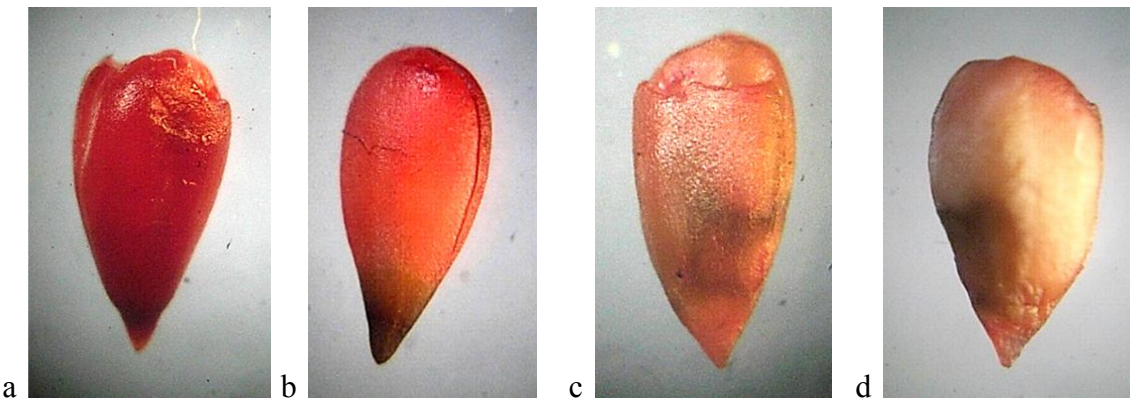


Figure 2.3.4: A normal viable seed stained uniformly (a), under-stained seed with discoloured radicle end (b), discolouration on cotyledon - radicle end appeared normal (c), and a non-viable seed with necrosis and shrivelled radicle end (d). (b) to (d) were assessed as abnormal.

2.4: Seed pre-treatment

For cool, moist pre-treatment of seeds, four replicates of fifty seeds were placed on two layers of 80 mm Advantec® filter paper in a standard plastic 90 mm petri dish and hydrated with 5 mL of deionised water before chilling. Petri dishes were sealed in ziplock plastic bags, and then placed in covered plastic containers. A 4 ºC refrigeration room was used when seeds required this treatment, usually referred to as cold stratification in scientific literature (Baskin, Baskin and Hoffman, 1992). The seeds were checked every third day for their state of hydration and microbial contamination. Where ethephon (2-chloroethylphosphonic acid) pre-treatment was required, the above procedure was repeated using 5mL of the required concentration of ethephon. Low temperatures did not appear to have any effects on imbibition (Appendix I).

2.5: Germination tests

Incubation of seeds was at a constant 25 ºC (±0.5) (as per ISTA,1999 for *E. purpurea*, Feghahatti and Reese, 1994) in an Axyos microdigital control Incubator with 12/12 h light/dark setting, with illumination provided by a bank of fluorescent tubes located at the rear of the cabinet. Where seeds had been pre-treated, they were moved to fresh filter paper with 5 mL of deionised water before incubation. Petri dishes were randomly placed in transparent covered plastic containers, each with a full complement of treatments, such that each container represented a block in the randomised block design. Extra deionised water was added to petri dishes as required. Germinated seeds (radical protrusion) were recorded and removed from the petri dish. Germination counts were done at the same time each day for fourteen days unless otherwise stated in the experimental chapters.

Where ethephon was used in pre-treatments, the seeds were transferred to fresh filter paper and petri dishes with 5 mL of water. Each petri dish was placed in an individual ziplock plastic bag to minimise the risk of cross contamination by ethylene gas emission.

2.6: Germination assessment

Radical protrusions of more than 1mm were assessed as having successfully completed germination. Seeds with malformed or discoloured roots were considered to be abnormal and not included in the germination assessment as germinated seeds. Germination capacity (GC) expressed as percentage germination, was the main parameter used when assessing seed germination. Mean germination time (MGT) and first day (FD) germination were also used where appropriate. GC is the maximum germination percentage (Bewley & Black, 1994) at termination of the trial and is calculated as:

GC = (number of seeds germinated / total number of seeds) x 100

For this study, the total number of seeds was the sum of germinated seeds plus the number of viable non-germinated seeds according to the squash and TZ tests.

The MGT is the mean time taken by individual seeds in a seedlot to complete germination (Bewley & Black, 1994).

$$MGT = \frac{\Sigma (t \cdot n)}{\Sigma n}$$

where t = time in days starting from day 0

n = number of seeds germinated on day t

The first day germination (FD) is the day on which the first appearance of a germinated seed within the replicates is observed.

2.7: The cut test

The cut (also known as squash) test is still an acceptable viability test (ISTA, 1996) but is purely observational and is subjective. Seeds that failed to complete germination were gently squashed with the flat of a scalpel blade to extrude the embryo which was examined for signs of discoloration, decay and fungal growth. Embryos that were firm and white were then TZ tested for viability.

2.8: Statistical analysis

Results were subject to analysis of variance using (ANOVA) the general linear models package of SPSS where appropriate. Where significant treatment effects were obtained, means were compared using Fischer's least significant difference (LSD), at P=0.05 after Steele and Torrie (1960). Results referred to as significant are at $P<0.05$ unless otherwise stated. Where shown, regressions were calculated using SPSS and error bars shown on graphs are standard errors of the mean in all cases. Residual plots from SPSS were checked for indications of non-normal data or heterogeneity of variance but no transformations were considered necessary. Zeros in the germination data were treated as missing plots, or where a whole treatment was involved it was deleted from the analysis as noted for some experiments.

Seed treatment experiments, particularly those involving temperature and time of exposure, present difficulties in experimental design and analysis. Because few laboratories have properly replicated controlled environment facilities, experimental designs are frequently pseudo-replicated and inappropriately subject to analysis of variance in which samples from a single chamber are treated as replicates. The general

issue has been discussed extensively by Hurlburt (1984), Gates (1991) and more recently, in relation, specifically, to seed experiments by Morrison and Morris (2000). In the present study, inclusion of a range of temperature and time of exposure treatments in experiments was essential to an improved understanding of germination characteristics of *Echinacea*, but access to controlled temperature facilities was limited and appropriate replication of four or five temperature treatments out of the question. Consequently experiments were arranged so that means of (usually) five germination plates at each temperature in a series could be analyzed in a linear regression, as suggested by Gates (1991), against germination to determine the temperature response. Where time of exposure was also included, regressions were calculated with time and temperature in a multiple regression. Because of difficulties in plotting three dimensional graphs and interpreting response surfaces, results have been presented as tables of means with standard deviations and the multiple regression equation noted in the relevant caption. Even in cases where the second variable makes little contribution to the coefficient of determination, the two variables have been retained in the equation and the relevant regression coefficients provide an adequate indication of the contribution of each variable. In experiments where one variable is replicated, and the other pseudo-replicated, separate analyses of variance were carried out for the replicated variable within each pseudo-replicated one (usually temperature).

In all experiments involving *in-vitro* pre-treatments, these were carried out in true replicates from the beginning of pretreatment to avoid the pseudo-replication issues discussed by Morrison and Morris (2000). All germination experiments with true replicates were randomized complete block designs with blocks arranged according to position in the germination cabinet.

Chapter 3: Understanding *E. angustifolia* seedlot variability

3.1: Introduction

In order to establish the germination characteristics of the *E. angustifolia* seedlots used in this study, preliminary germination tests were done to characterise the properties of the seeds. Investigations into seed responses to pretreatments were also performed in order to decide on the most appropriate methods to be used for the field experiment.

Cool, moist pre-treatment (generally referred to as cold stratification) of seeds has been found to reduce primary dormancy in *E. angustifolia* in previous studies (Feghahatti and Reese, 1994; Cover, 2004), and will be used on these seedlots to evaluate effectiveness. Traditionally, stratification involved burying seeds in layers of moist sand and peat moss (Bonner *et al*., 1974). A more convenient way, naked stratification, is now practised where seeds are soaked for a short period of time, drained, then stored in plastic bags/containers and chilled (bulk stratification); or allowed to sit on saturated filter paper (laboratory) for the duration of the cold treatment (Bonner *et al*., 1974; Jones and Gosling, 1990).

The duration of cold stratification with water necessary for *E. angustifolia* to achieve maximum germination capacity appears to vary in the published literature. Baskin *et al*., (1992) reported maximum or close to maximum germination after eight weeks of low temperature in moist sand and light. Feghahati and Reese (1994) reported no significant increases in germination after a two-week pre-chill period in the dark but significant increases in light. This suggested that light could have a synergistic effect which will shorten the time needed for complete germination. In contrast, Parmenter *et al*., (1996),

reported maximum germination after two to three weeks stratification in a dark coolstore. However, unrecorded light exposure, such as during periodic checks on the seeds, may have been sufficient to satisfy germination requirements.

Ethephon as a dormancy releasing agent has been described in the literature (Feghahati and Reese, 1994; Macchia *et al*., 2001). Pretreatment with ethephon was generally combined with cold stratification.

To facilitate mechanised sowings with seed drills, pre-treated seeds for commercial sowing are required to be dried back for handling purposes. Although quiescent seeds have moisture content between 5-15% (Bewley & Black, 1994), pre-treated seeds are only required to be dry enough to be free-flowing but not too fragile to be damaged in the sowing process. This chapter will describe the initial germination profiles and viability of the seedlots with and without pre-treatments, and will determine the drying time required to prepare seeds with a desirable water content for mechanised sowings.

3.2: Materials and Methods

The three *E. augustifolia* seedlots, SL2002, SL2003 and SL2004, were used in these experiments to determine:

- germination characteristics with and without pretreatments,
- the effects of seed sterilisation,
- and the time required for imbibed seeds to dry back to the original weight.

3.2.1: Germination profile

The germination pattern of the seedlots was determined using the following treatments:

- control
- cold stratification (chilled, moist pretreatment) with deionised water for 8 & 4 weeks
- cold stratification with ethephon for 2 weeks.

Seeds were pre-treated and germinated as described in the previous chapter.

3.2.2: Surface sterilisation of seeds

Fungal growth on *E. angustifolia* seeds incubated in petri dishes was evident in previous experiments (Cover, 2004) and it was not known if the microbial activities had an effect on germination capacity. To determine the effect of microbial growth on seed germination, four replicates of 50 seeds were washed in 50 mL of a 1% commercial (White King®) bleach solution (12% sodium hypochlorite) for 10 mins with constant stirring, then rinsed four times in deionised water.

3.2.3: Seed dry-back

To determine the dry-back time required, four replicates of 400 (SL 2003) seeds were weighed and placed in plastic containers and left to imbibe with 12 mL of deionised water for 24 hours. The seeds were then removed and placed on four layers of absorbent paper towels to remove any free water on the surface of the pericarp. Seeds were placed in a single layer in 100x100 mm pre-weighed plastic mesh bags and then reweighed. The bags were placed as a single layer on top of the wire rack of a custom-made dryer developed by a local seed company. The fan of the dryer was turned on half an hour beforehand to stabilise the temperature of the room (~18 ^{o}C). No heat was used in the drying process. The bags were weighed at 15 minute intervals for 60 minutes, then at 30min intervals until the original dry weight was attained. Bags were randomly replaced on the rack to minimise any influence of the airflow on the drying rate of the seeds.

3.3: Results and discussions

3.3.1: Germination profile

There was a difference between the germination profiles of SL2002 and the other two seedlots in the control treatment. There was a low percentage spread of the daily germination of SL2002 (Fig.3.3.1) and a low germination capacity (GC) of 37.5% compared to the SL2003 and SL2004 (83.6% & 93.9% respectively) even after taking into consideration that there was a lower viability (77.6%) in SL2002.

The mean germination time (MGT) of SL2002 of 8.01 days was nearly twice that of the other two seedlots, and SL 2002 also failed to reach the 50% germination percentile (t_{50}) which was between 3.0-3.5 days for the other two seedlots (Fig 3.3.2). These results, together with a longer first day germination (FD) of 4 days, may be indicative of a dormancy trait in SL2002. The cumulative percentage germination again demonstrated a different profile for SL2002.

Cold stratification treatments with water and ethephon resulted in improvement in the germination performance of all seedlots for all parameters. Some seeds in SL2003 and SL2004 germinated while still under treatment and had MGTs of less than one day (Table 3.3.1). The treatments had widened the temperature range for germination enabling some seeds to germinate at 4^{o}C while still undergoing cold stratification.

Synchrony of germination was evident in all seedlots but especially pronounced in SL2003 and SL2004 with most of the germination completed within two days (Fig. 3.3.3). Compared to ethephon pre-treatment, the four–week water-only treatment of SL2002 did not achieve a high GC, although it did improve germination percentage and

MGT. Eight weeks of water-only cold stratification, however, did improve the GC (Table 3.3.2) to a similar level as the ethephon treatment which is in agreement with literature and previous work (Baskin, Baskin & Hoffman, 1992; Cover, 2004).

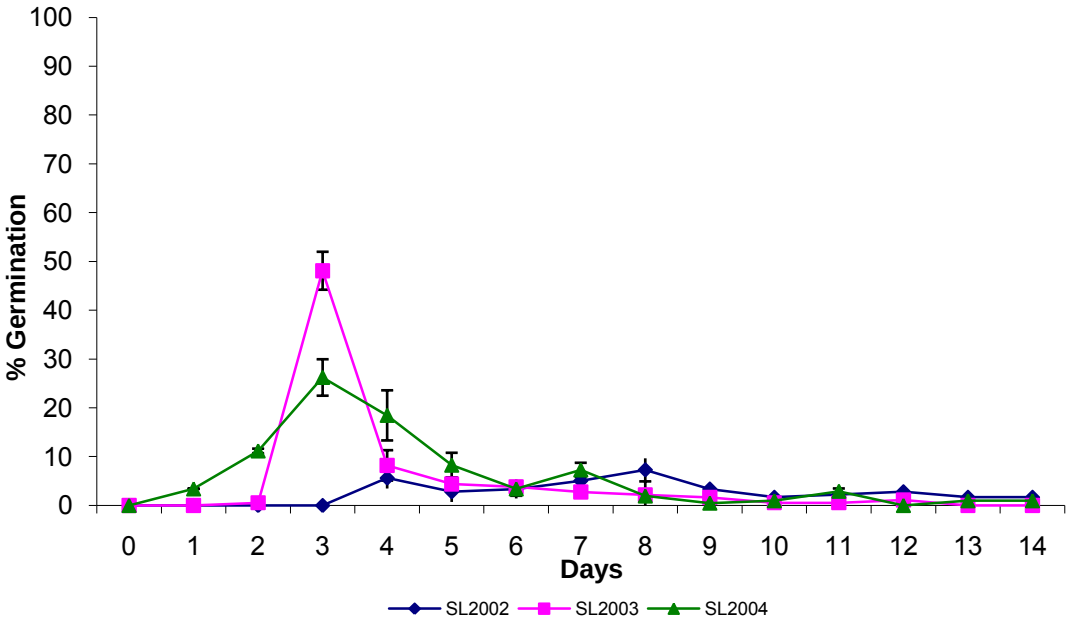


Figure 3.3.1: Daily germination profiles of untreated seedlots SL2002, SL2003 and SL2004, showing a low percentage spread over the 14 days for SL2002. Seeds were germinated at 25°C with 12/12 h light/dark conditions. Each value is the mean of four replicates. [Standard error bars are shown when larger than symbols].

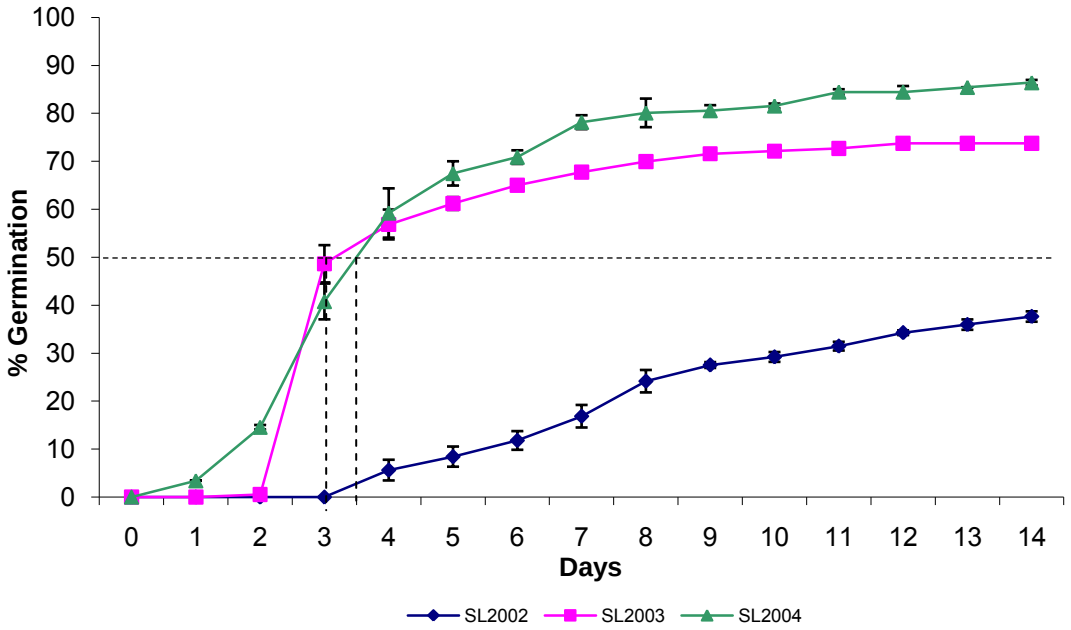


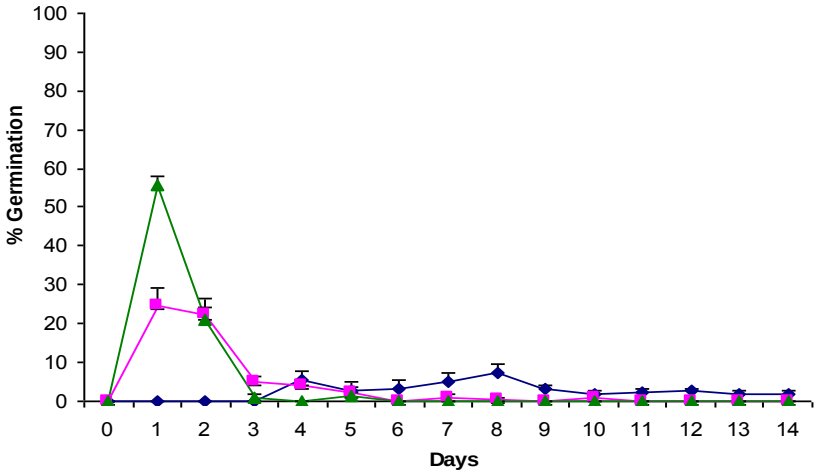
Figure 3.3.2: Cumulative germination percentage of seedlots SL 2002, SL2003 and SL2004. The t_{50} for SL2003 & SL2004 are 3.0 and 3.5 days respectively. SL2002 did not achieve 50% germination. Seeds were germinated at 25 °C with 12/12 h light/dark conditions. [Standard error bars shown where larger than symbols].

Table 3.3.1: Comparing three seedlots of *E angustifolia* after cold stratification treatments with water (eight weeks) or ethephon (two weeks) at 4°C. Seeds were germinated at 25°C with 12/12 h light/dark conditions

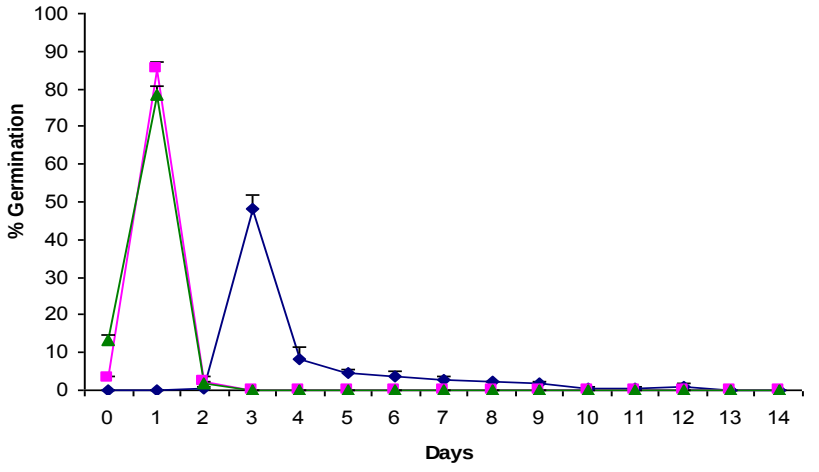
Seedlot	Treatment	%GC	% viability	FD	MGT
SL2002	water	60.81	80.96	4	4.51
	ethephon	79.02	83.80	1	1.37
	control	37.51	77.59	4	8.01
SL2003	water	90.98	92.00	0	0.99
	ethephon	93.78	93.78	0	0.88
	control	73.56	83.60	3	3.99
SL2004	water	91.45	93.46	0	0.98
	ethephon	93.40	94.93	0	0.97
	control	85.45	93.88	2	4.42

Table 3.3.2: Effect of cold stratification in water for four or eight weeks on a seedlot (SL2002) with a high proportion of dormant seeds. Seeds were germinated at 25°C with 12/12 h light/dark conditions.

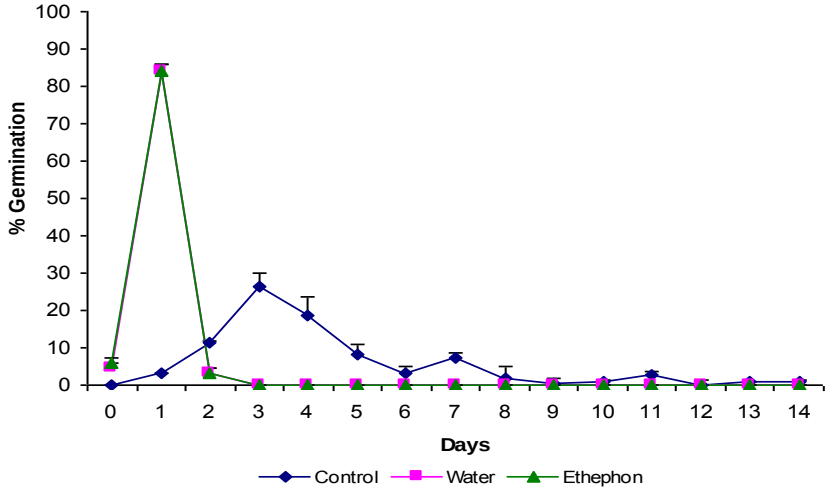
Seedlot	Treatment	%GC	% viability	FD	MGT
SL2002	4 weeks	60.81	80.96	4	4.51
	8 weeks	80.93	81.94	1	1.35
	control	38.52	79.34	4	7.56



A



B



C

Figure 3.3.3: Germination profiles of SL2002 (A), SL2003 (B) and SL2004 (C) with or without pre-treatments. Pretreatments with water was for 8 weeks and ethephon for 2 weeks at 4 ^{o}C. Germination was carried out at 25 ^{o}C, 12/12 h light/dark. Seeds were germinated at 25 ^{o}C with 12/12 h light/dark conditions. Each data set is the mean of four replicates. [Error bars are shown where larger than symbols].

3.3.2: Surface sterilisation of seeds

There was no obvious reduction in microbial growth between the sterilised seeds and the non-sterilised seeds after fourteen days. It is possible that the concentration used was insufficient to inhibit growth but as there was some reduction in the GC (Table 3.3.3), a higher concentration may have a deleterious effect on germination. Trace amounts of sodium hypochlorite had been found to interfere with protein uptake in metabolic studies (Abdul-Baki, 1974). Microbial growth was prevalent when the germination period was extended beyond 14 days. It was therefore possible to reduce any impact on germination results by terminating the testing period at two weeks instead of sterilising the seeds.

Table 3.3.3: Comparison between surface-sterilised and non-treated *E. angustifolia* SL2003. Seeds were germinated at 25 ^{o}C with 12/12 h light/dark conditions.

Seedlot	Treatment	%GC (14 days)	% viability	GC:viability ratio
SL2003	bleach	66.88	85.50	0.83
	water	72.51	89.58	0.84

3.3.3: Seed dry-back

From the graph (Fig. 3.3.4), the drying time required for the seeds to return to the original dry weight was ~105min, after which there was no further percentage change in weight. This has not taken into account the weight loss of solutes during the soaking process. The seeds were sufficiently free flowing after ninety minutes of drying and this time period will be used for the field trial. There was no significant difference in the GC

and viability between the wet seeds (67.9% and 76.0% respectively) and the dried seeds (69.0% and 78.64%).

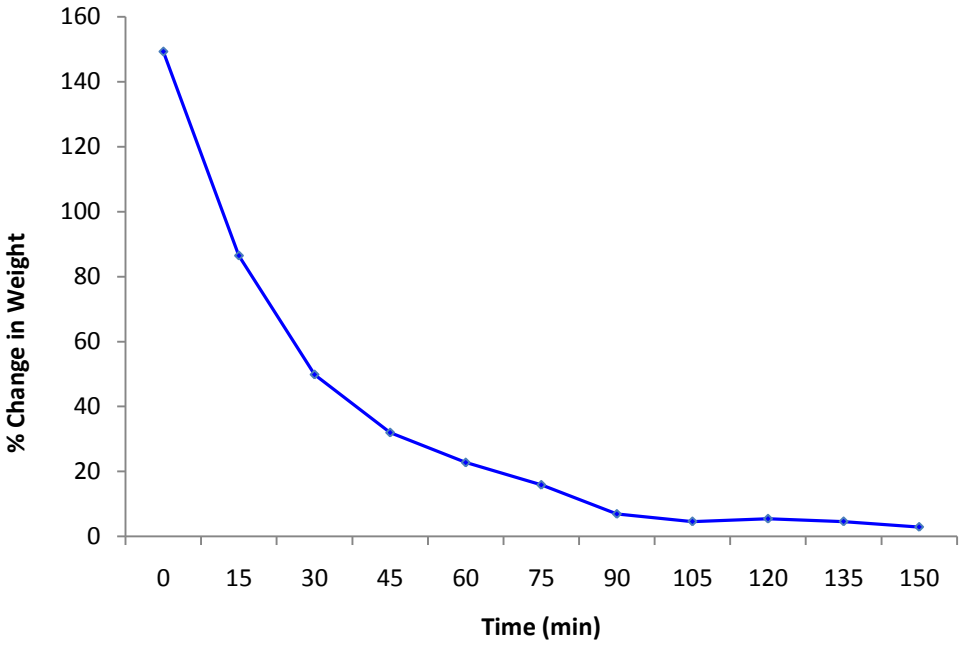


Figure 3.3.4: Percentage change in weight of SL2003 samples of 400 seeds each. The difference in weight was measured from fully imbibed seeds at zero time. Each data point is a mean of four replicates.

3.4: Summary

SL2002 demonstrated a different profile from the other two seedlots with a lower GC, a longer MGT and longer FD germination, but responded to pre-treatment with cold stratification especially with ethephon. This is indicative of a seedlot with a dormancy trait.

Cold stratification with both water and ethephon improved GC, FD and MGT on all three seedlots but was more marked in SL2002. Pre-treatments synchronised germination in all seedlots with most seeds germinating within two days. Ethylene release from ethephon is widely accepted as the trigger for dormancy release but its

action is unclear. Similarly, cold stratification is effective but the physiology is not entirely clear.

The dry back time required to achieve a free-flowing seedlot was approximately 90 minutes with a non-heated, fan-assisted drying rack at an ambient temperature of approximately 18 ^{o}C.

Chapter 4: Investigating primary dormancy in the *in vitro* germination of *E. angustifolia*

4.1: Introduction

The definition of seed dormancy is not unanimous among seed scientists. Although the literature on the subject is extensive, a detailed review is beyond the scope of the present study, and the discussion draws on earlier reviews, particularly those by Baskin and Baskin in 1998 and 2004, and literature cited therein. The review centres on classification of dormancy and practical implications for managing the germinating seed. Genetic control and the associated biochemistry and physiology of dormancy and dormancy release have not been included.

In another review, Vleeshouwers *et al*., (1995) mentioned Harper (1959), Vegis (1964), Bewley and Black (1982), Karssen (1982), Simpson (1990) and others who had, through the years, examined and elaborated on the concept of dormancy. Generally speaking, it is now acceptable to define seed dormancy as the failure of an intact viable seed to complete germination in the presence of favourable conditions such as adequate water, oxygen and temperature (Bewley and Black, 1982). However, this statement is not sufficient without stipulating an acceptable time period. To complicate matters, some dormant seeds can complete phase I and/or II germination (Fig.1.6.1) without progressing to post-germination Phase III and continued growth.

Dormant seeds are said to have an intrinsic block to germination, which may disappear slowly (after-ripening) or by the application of certain conditions to potentiate germination (Bewley and Black, 1982). It is difficult to pinpoint exactly when a

dormant seed makes the transition to non-dormant. In their 2006 review, Finch-Savage and Leubner-Metzger (2006) said that "definitions of dormancy are difficult because dormancy can only be measured by the absence of germination". Furthermore, individual seeds in a seed population can germinate over a range of conditions and this range can change over time, as in after-ripening (Baskin and Baskin, 1993; Finch-Savage *et al*., 2007; Schütz, *et al*., 2002); or the dormancy state can fluctuate, leading to dormancy cycling (Dunbabin and Cocks, 1999).

Not all seeds within a seedlot or even on the same seedhead have primary dormancy. Within a species known to have a dormancy trait, individual seeds within the one sample exhibit different degrees of dormancy. This variation is sometimes distinguishable by colour, size, shape or coat thickness, for example, smooth black seeds of *Chenopodium album* have the deepest dormancy compared with reticulated black, or reticulated or smooth brown seeds from the same plant (Bewley and Black, 1994). Similarly, *Bidens bipinnate* has outer, deeply dormant, short, brown and rugose cypselas, and longer, black, smooth inner ones. *Xanthium pennsylvanicum*'s dispersal unit contains an upper deeply dormant seed, and a lower less dormant seed. S*alsola komarovii* has dispersal units with either long-winged green seeds (non-dormant) or short-winged yellow seeds (dormant) (Yamaguchi *et al.*, 1990). Differences in the degree of dormancy can also be positional, such as in *Avena fatua* where the earliest ripening grains on the panicle are least dormant; or in *Aegilops ovata* where the heaviest grain from the lowest spike is non-dormant (Datta *et al*., 1972). Polymorphism is the term used originally to describe these morphological differences, but is now also applied to physiological dormancy variations. Some literature uses the term heteromorphism or heteroblasty to refer to the different dormancy states (Dekker, 2003;

Bewley and Black, 1982). This variation is, however, not evident in *Echinacea* species and cannot be used as a guide to a seedlot's dormancy status. Dormancy in *Echinacea* species varies markedly between seedlots with no apparent morphological differences, or identifiable or reported relationships with seed source or the parent's growing conditions.

4.1.1: Classification

Seed dormancy is classified as either primary or secondary (Crocker, 1916; Crocker and Barton, 1957; Villiers, 1972; Bewley and Black, 1982). Primary dormancy is an inherent trait at the time of dispersal and secondary dormancy is an environmentally induced state which is not present at dispersal. These two terms are variously referred to as endogenous and exogenous dormancy (Nikolaeva, 1977; Baskin & Baskin, 1998); or innate and enforced dormancy (Harper, 1965; Barton, 1965; Roberts, 1972). *E. angustifolia* is generally noted in the literature as having primary dormancy (Feghahati and Reese, 1994; Macchia *et al*., 2001) and the question of seedlot differences are not mentioned.

Primary dormancy is further categorised into different types. The simplest and most commonly used is that proposed by Bewley & Black (1994) who described two mechanisms, *coat-enhanced* dormancy, and *embryo* dormancy which may exist simultaneously or successively. Coat-enhanced dormancy can be a mechanical restraint, interference with water uptake, interference with gas exchange, light or prevention of inhibitors from exiting the embryo. Embryo dormancy may be due to inhibitors derived from cotyledons or more generally the embryo itself; and abscisic acid (ABA) is believed to be the major inhibitor involved (Chen *et al*., 2007; Feurtado *et al*., 2007;

Kermode, 2005). Earlier, Nikolaeva (1977) referred to embryo and coat-enhanced dormancy as *endogenous* and *exogenous* dormancy. Endogenous dormancy is further qualified as physiological (with a physiological inhibiting mechanism), morphological (underdeveloped embryo) or morpho-physiological (combination); and exogenous dormancy as physical (impermeability), chemical (inhibitors) or mechanical (structural). More recently, Baskin and Baskin (2004) proposed a modification of Nikolaeva's system extending the number of classes to five: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PD + PY). These classes are further subdivided into levels and types. PD contains three levels and five types, and MPD has eight levels and no types (Table 4.1.1). PD (non-deep) is the most common kind of dormancy. For ease of discussion, Baskin & Baskin's system will be used here to further explain the different types of dormancy.

Table 4.1.1: A classification system for seed dormancy (modified from Nikolaeva, 1977; Baskin & Baskin, 1998). This system does not include seeds with undifferentiated embryos (Baskin & Baskin, 2004).

A Class	-	*Physiological dormancy* (PD)
		3 Levels – deep, intermediate, non-deep
		Types – 1, 2, 3, 4 and 5 (of non-deep PD)
B Class	-	*Morphological dormancy* (MD)
		(does not include seeds with undifferentiated embryos)
C Class	-	Morphophysiological dormancy (MPD)
		8 Levels – non-deep simple, intermediate simple, deep simple, deep simple epicotyl, deep simple double, non-deep complex, intermediate complex and deep complex (see Table 3) (does not include seeds with undifferentiated embryos)
D Class	-	*Physical dormancy* (PY)
E Class	-	*Combinational dormancy* (PY + PD)
		1 Level – non-deep PD (probably both Type 1 and Type 2 are represented)

Physiological dormancy

Physiological dormancy is divided into three levels: non-deep, intermediate and deep. Non-deep is common in seeds of species from temperate zones and occurs in many weeds (cocklebur, henbane), crop plants (wheat, sunflower, lettuce), many garden flowers, and some woody plants. Seeds with non-deep physiological dormancy, when stored dry at room temperature, come out of dormancy (dry after-ripening) after a period of time. This time can be shortened by a short period of cold stratification (e.g. *Digitaria ischaemum* seeds need eight weeks stratification or one year dry after-ripening (Toole and Toole, 1941 as cited in Baskin and Baskin, 1998). Another feature of seeds with non-deep PD is their response to chemicals treatment. Ethylene, potassium nitrate, kinetin, gibberellins (GAs) are some of the more common treatments used for dormancy release (Baskin and Baskin, 1998; Chuanren *et al*., 2004). Light requirement has been reported as a manifestation of non-deep PD (Nikolaeva, 1977) although species differ in when the light is required, or may also lose the need after dry after-ripening or if otherwise pre-treated. Another characteristic of non-deep PD (Type 1-3 in Table 4.1) is dormancy-cycling whereby the seeds go through a continuum of dormancy states and, consequently, sensitivity to factors such as temperature, light and/or growth regulators. The word ‚conditional' dormancy is used to describe the transitional state between dormancy and non-dormancy when the seeds germinate over a narrow range of conditions (Baskin & Baskin, 2001).

Covering structures (endosperm or cotyledons) are also believed to be another cause of non-deep PD. In contrast to *physical* dormancy (PY), there is usually no restriction of water movement into seeds (Roberts, 1961; Dungey and Pinfield, 1980). However, covering structures can restrict oxygen movement into the embryo or contain

compounds that fix oxygen making it unavailable to the embryo (Barthe *et al*. 2000; Benech-Arnold *et al*. 2006). Embryo covers could also contain inhibitors or prevent leaching of inhibitors from the embryo (Black and Wareing, 1959; Nikolaeva, 1977; Baskin and Baskin, 1998; Bewley and Black, 1994). Physical restriction is also linked to embryo growth potential, or the capacity of the embryo to penetrate the constricting structure. Embryos of non-dormant seeds of *Xanthium pensylvanicum* were found to have twice the thrust of dormant seeds (Esashi and Leopold, 1968). Various pretreatments such as cold stratification, temperature, or light/dark (Baskin and Baskin, 1998) have been shown to increase growth potential which is presumably related to osmotic potential of the embryo and hence the turgor pressure generated as hydration proceeds. The alternative to increasing the capacity of the embryo to penetrate a restricting layer is for the layer itself to become weaker (da Silva *et al.,* 2004) The endosperm has been implicated as the main restricting force to radicle protrusion and enzyme activity (endo-β-mannanase, β-1,3-glucanase, β-mannosidase) associated with cell wall hydrolysis, has been linked to endosperm breakdown at about the time of radicle emergence (Leubner-Metzger *et al*., 1996; Bewley, 1997; Dutta *et al*., 1997; Leubner-Metzger, 2003).

The hormone balance hypothesis, which focused mainly on the antagonistic interactions of GA (promoting germination) and ABA (imposing dormancy), has been studied by many researchers (Karssen and Lacka, 1986; Grappin *et al*., 2000, Kucera, *et al*., 2005). GA's role is in promotion of germination and the release of dormancy. ABA, as the positive regulator of dormancy induction, can be produced during seed development or maturation by the embryo itself or the maternal tissues. However, only the embryonic ABA imposes and maintains dormancy (Karssen *et al*., 1983; Hilhorst and Karssen,

1992, Toorop *et al*., 2000; Leubner-Metzger, 2003). In their summary, Finch-Savage and Leubner-Metzger (2006) suggested that a dormant embryo has a high ABA:GA ratio, high ABA sensitivity and low GA sensitivity; and dormancy release involves „remodelling of hormone biosynthesis and degradation' to achieve a low ABA:GA ratio.

ABA and GA are not the only hormones involved in dormancy regulation and germination. Ethylene has been shown to interfere with ABA signalling and counteract ABA effects on germination (Esashi, 1991; Kepczynski and Kepczynska, 1997; Matilla, 2000; Feurtado and Kermode, 2007) and has been shown to synchronise and improve germination in *E. angustifolia* (Macchia *et al*., 2001; Sari *et al*., 2001; Cover, 2004.). Brassinosteroids, cytokinins (Pence *et al.*, 2006) and auxins also promote germination in some species (Black *et al*; 1974; Feurtado and Kermode, 2007).

Temperature and light are factors inextricably tied with dormancy release, although not all species require light for germination. As mentioned earlier, although species-dependent, physiological dormancy can be broken by warm or cold stratification (Table 2) and an ideal temperature range is necessary for successful germination and growth (Bouwmeester and Karssen, 1992; Probert, 2000; Batlla and Benech-Arnold, 2003). As a general rule, embryos isolated from seeds with intermediate PD usually show normal growth. Such seeds require a prolonged cold stratification (two to three months) or treatment with GA can be substituted for cold stratification in some species (Baskin and Baskin, 1998), although germination may only occur under specific conditions, for example, *Acer negundo* seeds require removal of the pericarp for a positive GA effect (Nikolaeva, 1977). In contrast, seeds with deep PD typically have embryos which will

not grow normally if removed from the seed and require prolonged cold stratification to break dormancy. Many tree species (mountain ash, maple and quince) have deep PD, and exogenous GA had no effect on dormancy in the intact seeds (Nikolaeva, 1977). The three types of PD are best summarised by Baskin and Baskin (1998) in Table 4.1.2 below.

Table 4.1.2: Characteristics of dormancy in seeds with deep, intermediate and non-deep physiological dormancy (from information in Baskin & Baskin, 1998)

Deep	Excised embryo produces abnormal seedling
	GA does not promote germination
	Seeds require 3–4 months of cold stratification to germinate
Intermediate	Excised embryo produces normal seedling
	GA promotes germination in some (but not all) species
	Seeds require 2–3 months of cold stratification for dormancy break
	Dry storage can shorten the cold stratification period
Non-deep	Excised embryo produces normal seedling
	GA promotes germination
	Depending on species, cold (≤ 0–10 °C) or warm (≥15 °C) stratification breaks dormancy
	Seeds may after-ripen in dry storage
	Scarification may promote germination

Morphological dormancy

Seeds of some species with MD have differentiated (distinguishable cotyledons and hypocotyl/radicle), but underdeveloped embryos and embryo growth is required before germination occurs. In other species where the embryo is not differentiated, both differentiation and growth have to take place first. For example, seeds of *Conium*

maculatum with MD can also develop PD when exposed to changes in the environment (Baskin & Baskin, 1990).

Morphophysiological dormancy

Seeds with MPD cannot germinate until the embryo has grown to a species-specific size and the PD of the embryo broken. Some require a combination of warm and cold stratification to germinate (Baskin & Baskin, 1998; Nikolaeva, 1977). There are eight levels of MPD (Table 5.1) and each requires different stratification temperatures or combinations to break dormancy (Baskin & Baskin, 2001). An example of this is *Paeonia suffruticosa* where the radicle emerges in autumn with temperatures of 12.5 to 21^{o}C, but the epicotyl requires a period of between 1 to 10^{o}C before emerging in spring (James *et al*., 1996; Baskin and Baskin, 2001). This was termed „epicotyl dormancy' by Barton in 1965.

Physical dormancy

PY is primarily due to impermeability of the seed or fruit coat to water, usually because of the presence of one or more layer of impermeable palisade cell layer. Sclereid cells (most commonly macrosclereids) of the palisade layer have thick lignified secondary walls which contain water-repellent substances such as cutin, lignin, suberin, waxes and others (Rolston, 1978). Seeds of the *Rhus* species have PY due to a water-impermeable endocarp but the impermeability varies at species and seedlot levels (Li *et al*., 1999). PY is known to occur in nine orders and fifteen families of angiosperms (Baskin, Baskin and Li, 2000), and is well represented in the *Fabaceae* family (Funes and Venier, 2006).

Combinational dormancy

PY+PD seeds may have both water-impermeable seed or fruit coats and physiologically dormant embryos and require both to be broken before germination can take place. The presence of a hard woody pericarp, such as the stone in *Prunus* spp, is a constraint but often a physiological dormancy barrier is also present.

Germination inhibitors are also implicated in some species. This was termed „chemical dormancy' by Nikolaeva (1977). Germination can occur after the pericarp is removed or the fruit is leached. However, it is uncertain in this case whether the actual germination unit is dormant or just quiescent. Most seeds with „chemical' dormancy are from tropical/subtropical regions.

4.1.2: Summary of literature review

From the literature review, dormancy is an adaptive trait with a temporal and/or spatial function. Such a trait delays its germination until environmental conditions are suitable for seedling growth, for example, in spring, or until the seeds are brought up to the soil surface from an unfavourable depth. *E. angustifolia* fits in the non-deep physiological dormancy (PD) class although the dormancy status appeared to be seedlot dependent (Chapter 3). Although not highly desirable in agricultural crops and plantation trees, primary dormancy can be a useful trait in some grain crops to reduce vivipary. In *E. angustifolia*, it is desirable in its wild state but not so in cultivation.

There are two forms of dormancy, primary which is intrinsic, and secondary which is induced. *E. angustifolia* appears to have primary dormancy initially but secondary dormancy may be induced when conditions are unfavourable.

There are several inter-related factors – hormones, enzymes, temperature, light, which determine dormancy release. Non-deep PD can be broken by seed treatment such as cold stratification and/or chemical means, and *E. angustifolia* had responded well to cold stratification and ethephon treatments (Chapter 3).

From an industry perspective, reducing the time of seed pre-treatment is advantageous as it minimises the danger of loss if there is a breakdown in the treatment process. The experiments in this chapter will endeavour to determine if lower or higher stratification temperatures, with or without ethephon, will initiate dormancy release in *E. angustifolia*; and whether there are ongoing toxicity effects from the application of ethephon. Previous work (Cover, 2004), indicated that an ethephon concentration of 10^{-3} M at 4 ^{o}C for 2 weeks was the optimum conditions for *E. angustifolia*.

The experimental questions for this chapter are:

- although the species experiences snow cover in winter and the seeds are exposed to around 0^{o}C for dormancy release, will even lower temperatures reduce the stratification time needed to break dormancy
- as ethylene released from ethephon is considered the dormancy releasing agent, would higher temperatures be more time-effective in reducing the stratification period,
- and does ethephon have an ongoing toxic effect on the growth of seedlings.

4.2: Materials & methods

Based on the results obtained in Chapter 3, seedlot SL2002 was used in these experiments, as it contained the highest percentage of dormant seeds. Seeds were stored at 4 ^{o}C between experiments in Chapters 3 and 4 for three weeks.

Three experiments were carried out. The first two examined *in vitro* germination in response to (1) stratification at a range of low temperatures (4 ^{o}C to –18 ^{o}C) for four different time periods (weeks) and (2) stratification with ethephon at a range of higher temperatures (4 ^{o}C to 37 ^{o}C) for four different time periods (hours). The third experiment (3) was an evaluation of toxic effects of ethephon seed treatment on subsequent seedling growth and development.

As for all other germination experiments, plots were separate samples of fifty seeds selected at random from the bulk stock of seed. All plots were then treated separately through to the completion of germination, with each time/temperature treatment replicated in separate petri dishes. Available facilities did not allow the controlled temperature environments to be replicated. Germination conditions and counts were as described previously.

Germination counts were used to calculate final germination percentage and, in Experiments 1 and 2, mean germination time (MGT) after Bewley and Black (1984). These figures were then used in the regressions (Experiments 1 and 2) or analyses of variance (Experiment 3) to estimate treatment effects. In Trial 3 individual treatments were compared using least significant difference (Steele and Torrie, 1980). Although set out as randomised block designs, experiments 1 and 2 were analysed using regression to

avoid problems with pseudo-replication of the temperature „treatments' (Gates, 1991) as discussed in the General materials and methods (Chapter 2). The regression analyses were calculated using the mean figures for each set of five „replicates' and the analysis was a multiple regression using temperature and time as the two independent variables in SPSS. Days to first germination were recorded in the three experiments but rapid germination resulted in numbers with high heterogeneity of variance and no statistical analyses were carried out.

Experiment 1: Low temperature water stratification

Seeds were stratified in water on filter papers in petri dishes at 4, 2, -8, -18 ºC for 1, 2, 4 or 8 weeks before germination. An untreated, (non-stratified) control treatment stored at the same temperatures was also included in the trial. The start of each of the time treatments was staggered to enable simultaneous incubation for germination. There were five replicates. At the conclusion of treatments, treated seeds were transferred to petri dishes with freshly moistened filter paper and allowed to germinate under the conditions described in Chapter 3. As noted above the results were analysed as a multiple linear regression using „Systat'. Mean and standard deviation for the control are given with the results table.

Experiment 2: High temperature ethephon stratification

Five replicates of seeds were stratified in 5mL of 10^{-3} M aqueous ethephon on filter paper in petri dishes at 4, 10, 25, 37 ºC for 6, 24, 48 or 72 hrs with an untreated (non-stratified control) treatment included in the experiment at incubation. Pre-treatments were again staggered for simultaneous incubation. At the conclusion of treatments, treated seed was transferred to petri dishes with freshly moistened filter paper and

allowed to germinate under conditions described above. As for the previous experiment the results were analysed as a multiple linear regression using „Systat'. Means and standard deviations are given with the results table.

Experiment 3: Glasshouse evaluation of ethephon toxicity

Samples of sixty seeds were treated with 5 mL of ethephon at concentrations of 10^{-2} M, 10^{-3} M or 10^{-4} M for 2 weeks. A control treatment was stratified in water only for eight weeks based on previous results (Cover, 2004). After pre-treatment, five seeds of each ethephon treatment were selected at random and planted 10mm deep into a standard (commercial) potting mix in 80mm plastic pots. Ten untreated seeds were also planted as controls. The remaining treated seeds were transferred to petri dishes for in vitro germination tests as described. The experimental design for *in-vitro* germination was four pretreatment concentrations and five replicates in a randomised block design blocked according to incubation chamber position. Results for analysis were final germination and viability percentages. The design for growing seedlings in the glasshouse was similar with untreated seeds included as a fifth treatment and pots blocked according to position in the glasshouse. Growth was monitored at 14 day intervals until 140 days when seedlings were carefully harvested and total length (root plus shoot) measured, before they were dried to constant weight and final weight recorded.

The glasshouse temperature was set at a constant 22 ^{o}C with natural daylength. Pots were watered by hand as required. Results were subject to analysis of variance and means compared using Fisher's least significant difference.

4.3: Results

Mean germination in the untreated control seeds was 35.2% (sd=9.8) with a viability of 74.0% (sd=7.5). The first day (FD) germination (appearance of the first germinant) was on day 3 with a mean germination time (MGT) of 7 days.

Experiment 1: Low temperature water stratification

Lower imbibition temperatures progressively impaired both germination and viability, with time of exposure having little effect. Mean germination and viability for each time/treatment combination and the regression equations are given along with untreated control results in Tables 4.3.1 and 4.3.2. For the four stratification temperatures and times, there was a significant ($P<0.001$) multiple linear regression with final germination percentage, accounting for 80% of the variation ($r^2=0.8$). Seeds stratified for eight weeks at 4 ^{o}C had a germination percentage of 63%, markedly higher than all other treatments and a notable outlier from the regression. Viability gave a similar regression ($P<0.001$) with an r^2 of 0.97. There were no notable outliers (Table 4.3.2).

The linear multiple regressions of temperature and time against final germination and viability were both significant ($P<0.001$). Mean germination time (MGT) plotted against temperature and time (Table 4.3.3) was also significant ($P=0.002$), but weaker than either percentage germination or viability ($r^2=0.62$). Again there were no notable outliers, but the untreated control had a longer mean germination time than all other treatments. As for the two previous regressions, the higher regression coefficient for temperature indicated a greater influence of temperature relative to time of exposure. First day germination (FD) was delayed as the temperatures decreased (Table 4.3.3).

Table 4.3.1: Percentage germination for *E. angustifolia* SL2002 at different stratification temperatures in water for different lengths of time. The regression equation (see text) relating time and temperature, %germination = 34.7 - 0.093 (weeks) + 1.3 (temperature), was significant at P<0.001 with an r^2 of 0.6. Subscripts in brackets are standard deviation of means.

	Stratification Temperatures (^oC)			
Stratification (weeks)	4	2	-8	-18
8	62.72 (13.1)	31.63 (7.6)	19.63 (4.7)	1.51 (2.0)
4	46.01 (9.4)	28.58 (4.1)	22.00 (9.1)	12.38 (8.9)
2	39.80 (15.0)	38.98 (21.5)	18.16 (7.0)	12.11 (4.4)
1	31.31 (4.0)	35.11 (19.2)	17.82 (8.1)	1.01 (1.2)
0	35.54 (11.1)	35.39 (9.5)	35.82 (10.2)	33.94 (8.5)

Table 4.3.2: Percentage viability for *E. angustifolia* SL2002 at the end of 2 weeks incubation at different stratification temperatures in water for different lengths of time. The regression equation (see text) relating time and temperature, %viability = 73.33 – 1.796 (weeks) + 2.39(temperature), was significant at P<0.001 with an r^2 of 0.73. Subscripts in brackets are standard deviation of means.

	Stratification Temperatures (^oC)			
Stratification (weeks)	4	2	-8	-18
8	82.4 (6.9)	74.3 (3.3)	44.8 (3.7)	4.5 (4.1)
4	74.0 (9.3)	69.0 (12.7)	40.6 (9.3)	17.4 (10.9)
2	80.0 (2.9)	68.5 (7.3)	40.3 (6.1)	15.2 (2.6)
1	78.6 (4.5)	70.8 (10.7)	52.0 (8.8)	11.5 (3.7)
0	77.7 (7.6)	72.4 (9.0)	72.2 (10.2)	73.9 (3.2)

Table 4.3.3: The mean germination time (MGT) and first day germination (FD) in days for *E. angustifolia* stratified at different temperatures in distilled water for different length of time. The regression equation (see text) relating time and temperature, MGT = 3.97 - 0.061(weeks) – 0.156 (temperature), was significant at P=0.002 with an r^2 of 0.6. Subscripts in brackets are standard deviation of means.

	Stratification temperature (^OC)							
Stratification (weeks)	4		2		-8		-18	
	MGT	FD	MGT	FD	MGT	FD	MGT	FD
8	1.84 (0.31)	1	2.49 (0.29)	1	6.36 (0.29)	4	6.33 (0.35)	4
4	2.19 (0.73)	1	3.16 (1.00)	1	6.75 (1.55)	4	5.64 (0.84)	3
2	3.45 (0.50)	1	3.69 (0.75)	1	6.67 (0.88)	4	5.92 (0.72)	3
1	2.84 (0.51	2	3.39 (1.28)	2	6.86 (0.72)	4	5.00 (1.41)	4
0	7.19 (0.99)	3	7.25 (1.07)	3	7.25 (0.95)	3	7.25 (1.06)	3

Experiment 2: High temperature ethephon stratification

The highest stratification temperature markedly reduced both germination and viability at times of 24 hour or longer, but there was little response to temperature at 25 ^{o}C or lower for any of the times tested. There were significant (P<0.001) multiple linear regressions between time and temperature and both germination percentage and viability (r^2 = 0.47) (Tables 4.3.4 and 4.3.5). Although significant (P=0.02) the MGT regression against temperature and time was weaker accounting for only 45% of the variability (r^2=0.45) (Table 4.3.6). Similarly, the regression for percentage first day germination (Table 4.3.7) was weak, accounting for only 40% of the variability, although significant at P=0.04.

Table 4.3.4: Percentage germination of *E. angustifolia* SL2002 stratified at different temperatures and times. The regression equation (see text) relating time and temperature, %germination = 76.1 - 0.125(hours) – 0.77 (temperature), was significant at P=0.016 with an r^2 of 0.47. Subscripts in brackets are standard deviation of means.

	Stratification temperatures (^{o}C)			
Stratification time (hrs)	37	25	10	4
72	20.8 $_{(12.9)}$	67.9 $_{(18.9)}$	68.0 $_{(7.6)}$	57.4 $_{(13.8)}$
48	24.0 $_{(8.0)}$	65.8 $_{(5.8)}$	68.2 $_{(8.2)}$	62.4 $_{(6.1)}$
24	37.7 $_{(9.4)}$	66.6 $_{(4.1)}$	61.9 $_{(14.1)}$	54.9 $_{(9.1)}$
6	53.9 $_{(7.4)}$	61.3 $_{(3.8)}$	64.4 $_{(7.4)}$	72.9 $_{(9.5)}$

Table 4.3.5: Percentage viability at the end of a two-week incubation period of *E. angustifolia* SL2002 stratified at different temperatures and times, %viability = 76.1- 0.125(time) - 0.77(temperature). The regression is significant at P=0.16, but fairly weak with an r^2 of 0.47. Subscripts in brackets are standard deviation of means.

	Stratification temperatures (^{o}C)			
Stratification time (hrs)	37	25	10	4
72	22.3 $_{(14.2)}$	72.8 $_{(15.1)}$	71.4 $_{(7.2)}$	62.8 $_{(13.9)}$
48	27.0 $_{(8.3)}$	70.7 $_{(7.6)}$	72.2 $_{(9.6)}$	68.3 $_{(5.8)}$
24	44.8 $_{(9.8)}$	70.6 $_{(3.8)}$	66.9 $_{(6.7)}$	69.1 $_{(12.4)}$
6	62.2 $_{(9.2)}$	69.8 $_{(4.1)}$	71.4 $_{(10.6)}$	76.8 $_{(6.6)}$

Table 4.3.6: The mean germination time (MGT) and for *E angustifolia* SL2002 stratified at different temperatures in 10^{-3}M ethephon for four different period of time. The regression equation (see text) relating time and temperature, MGT = 2.93 - 0.008(weeks) – 0.15 (temperature), was significant at P=0.02 with an r^2 of 0.45. Superscripts in brackets are standard deviation of means.

	Stratification temperatures (°C)			
Stratification time (hrs)	37	25	10	4
72	2.32 (0.46)	1.98 (0.26)	1.82 (0.23)	2.77 (0.25)
48	2.11 (0.19)	1.78 (0.31)	1.95 (0.34)	2.74 (0.25)
24	2.12 (0.08)	1.94 (0.14)	2.49 (0.20)	2.79 (0.23)
6	2.54 (0.16)	2.56 (0.33)	2.98 (0.21)	2.95 (0.15)

Table 4.3.7: First day germination (FD) in days and percentage germination on FD (%FD) for *E angustifolia* SL2002 stratified at different temperatures in 10^{-3} M ethephon for four different period of time. The regression equation (see text) relating time and temperature, FD = -2.34 + 0.342(weeks) + 0.51 (temperature), was significant at P=0.037 with an r^2 of 0.4.

Stratification time (hrs)	Stratification temperature (°C)							
	37		25		10		4	
	FD	%FD	FD	%FD	FD	%FD	FD	%FD
72	1	21.95	1	46.15	1	40.00	1	5.41
48	1	19.15	1	53.47	1	36.72	1	4.69
24	1	26.67	1	36.57	1	5.65	2	1.79
6	1	10.81	1	11.48	1	2.31	2	0.00

Experiment 3: Glasshouse evaluation of ethephon toxicity

There was a significant (P<0.001) treatment effect on germination *in vitro* (Fig. 4.3.1), with the untreated control having a markedly lower germination than all other treatments. There was also a significantly (P<0.05) higher emergence from the 10^{-2}M ethephon treatment compared with the other pretreated seeds. There were no treatment effects on viability *in vitro* with a mean of 79.0% (sd=2.7). Overall, glasshouse emergence did not reflect percentage germination under *in vitro* conditions with a 14-50% difference across the treatments (Figure 4.3.1). The preteated seeds had mostly

completed germination (*in vitro*) in two days with germinants appearing on day one (FD) except for the highest concentration, 10^{-2}M ethephon–treated (E2), which had FD on day two. Glasshouse emergence with E2 had the best emergence of 44% compared to the other pre-treated seeds of ~26% on day ten. Untreated seeds did not achieve 20% emergence till day fifty-two. The MGT (emergence) for the pretreated seeds of 32% compared to 41% again indicated the effectiveness of the treatments.

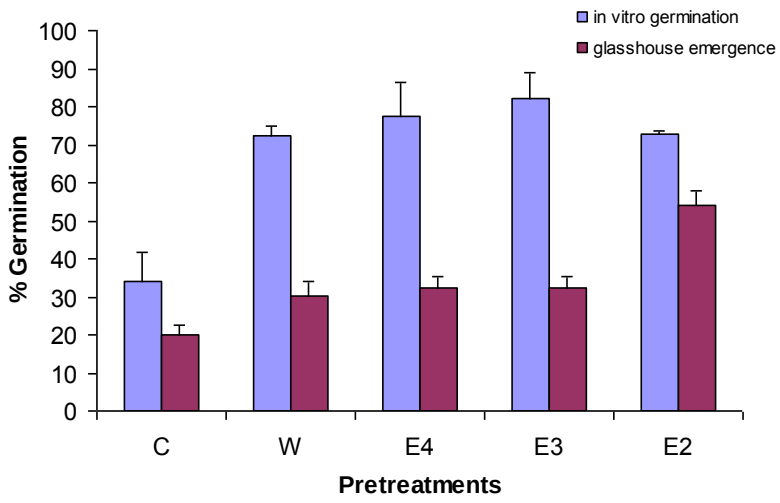


Figure 4.3.1: Comparing *in vitro* germination with glasshouse emergence of *E. angustifolia* SL2002. There was a significant treatment effect in both trials and the LSD for comparison between means within each trial is 9.2 and 10.8 respectively. [Pretreatments: C=control; W= water-stratified (8 weeks); E4 to E2= ethephon 10^{-4}M to 10^{-2}M].

Visually, seedlings from the highest concentration of ethephon were stunted initially but appeared to regain normality by the end of two weeks, and were of similar size to the other treatments. 'There were significant treatment effects on length (P=0.046) and shoot number (P=0.028), but no effect on weight (P=0.25) or leaf number (P=0.36). When measured at 140 days there was a significant (P=0.046) treatment effect on length with the highest ethephon treatment showing a significantly reduced length compared with the untreated control or water only. There was no significant treatment effect on

the final weight of plants (P=0.25). The number of reproductive shoots was also lowest for E2 with a mean of 1.5 compared with 2.8 for the other concentrations, and 3.8 in the water pretreatment. Leaf numbers were also lower for E2 with a mean of 11 compared with 15 in the other treatments (Table 4.3.8).

Table 4.3.8: Final growth assessment of *E. angustifolia* SL2002 140 days from sowing. There were significant treatment effects on length (P=0.046) and shoot number (P=0.028), but no effect on weight (P=0.25) or leaf number (P=0.36). E = ethephon concentrations: E4=10^{-4} M, E3=10^{-3} M, E2=10^{-2} M.

Treatment	Length(mm)	Dry Weight(g)	# Shoots	# Leaves
Water	497	1156	3.8	14.5
E4	490	914	2.8	15.1
E3	475	778	2.8	14.9
E2	432	822	1.5	11.0
LSD	*36.9*	*Ns*	*1.13*	*ns*

4.4: Discussion

Stratification temperatures below 4 ^{o}C did not improve germination percentage or reduce the length of stratification required for improved germination. Viability of the seed was also compromised as the temperatures decreased. This initially appeared surprising given that, in its natural habitat, seeds are subjected to snow cover in winter. There is, however, evidence that soil under snow cover is warmer, has less variability and may have temperatures above 0^{o}C (Riseborough, 2001; Decker *et al.*, 2003). Bleak (1959) found that soil temperature at a depth of 0.5 inch (12.7 mm) was only 32.5 ^{o}F (-0.2 ^{o}C) under an average of 46 inches (1168.4 mm) of snow whereas snow-free areas had soil temperatures below freezing. The ability of the *E. angustifolia* seeds to withstand 2 ^{o}C for two to eight weeks, demonstrated here, without detrimental effects on germination and viability, suggested that seeds can be sown in late autumn or winter

as long as the below-surface soil is not frozen for extended periods. Sub-surface soil temperatures below 0 ^{o}C are rarely recorded in cropping areas in Tasmania, and this early sowing will allow non-treated seeds to undergo a natural stratification in field.

It is possible that the seeds may withstand even lower temperatures (below 0 ^{o}C) as there was some germination even after 8 weeks of stratification at -18 ^{o}C. Similar low temperature tolerance was demonstrated for conifer seeds by Hawkins *et al*., (2003), citrus seeds (Horanic and Gardner, 1958) and clover seeds (Steinbauer, 1926), provided the low temperature period is not extended. Such low temperatures are not an issue for field planting in Tasmania and do not offer any advantages as a pre-treatment as the loss of seed viability even after one week at -18 ^{o}C was significant.

Higher temperatures with ethephon treatment did not improve germination, but there was also no loss of viability or reduction in germination across the range 4 ^{o}C to 25 ^{o}C for 6 to 72 hours. Thus seeds can be stratified in 10^{-3} M ethephon at ambient temperature for six hours on the day of sowing, therefore eliminating stratification for longer periods with associated risks. It also indicates that controlled temperature conditions are not required and stratification can be carried out in the usual range of ambient temperatures occurring in Tasmania.

The markedly different germination results between the *in vitro* germination tests and germination in the glasshouse are difficult to explain. There is a possibility that conditions in the glasshouse caused a reversion to dormancy, as the percentage emergence of seeds pretreated with water or the lower concentrations of ethephon was very similar to the control. Pretreatment with the highest concentration of ethephon,

resulted in a significant increase in emergence, suggesting that the dormancy breaking effect of ethephon at 10^{-2} M is physiologically different to the effect of water and the lower ethephon concentrations effective on *in vitro* germination. If, as appears to be the case, conditions in the glasshouse soil resulted in a return to dormancy, it was only the highest ethephon concentration that promoted (under these conditions) irreversible breaking of dormancy.

From the results, there is no indication of what the soil factors were that promoted a return to dormancy may have been. Temperature and water potential in the soil would both have been more variable than in petri dishes in a growth cabinet. Although the previous experiment showed that the effectiveness of 10^{-2} M ethephon is not sensitive to temperature during stratification, longer term exposure to diurnal temperature or water potential fluctuations may induce a return to a more dormant state. There is no indication from the results whether the more variable conditions of a field sowing would reverse the 10^{-2} M ethephon effect shown here.

The growth results indicated a near 30% reduction in plant length after seed pretreatment with 10^{-2} M ethephon, persisting for nearly five months after planting. There was no corresponding significant effect on plant weight, but results were highly variable. It appears then that this ethephon treatment balances a 30% reduction in growth against a 40 - 50% increase in germination under the conditions of this trial. Under field conditions, where conditions are likely to be more variable and perhaps more extreme, growth and germination responses may change in different ways. The use of 10^{-2} M ethephon and its effect on both germination and subsequent growth needs further investigation.

4.5: Summary

Below 4 ^{o}C, stratification temperatures did not improve germination percentages or reduced the length of stratification required for improved germination. Viability was compromised as the temperature decreased to -8 ^{o}C and below.

Although ethephon treatment at temperatures above 4 ^{o}C did not improve germination significantly, germination percentages for 6-72 hours (except for 37^{o}C) were comparable to seeds stratified in water for eight weeks. It may therefore be possible to treat seeds with ethephon for a period of six hours on the day of sowing to promote more acceptable levels of germination under field conditions.

There was some growth reduction in ethephon-pretreated plants but the improved emergence may outweigh the negative effects on growth especially with concentrations of 10^{-3} M and less.

Chapter 5: Field germination of *Echinacea angustifolia*

5.1: Introduction

Dormancy within *E. angustifolia* seedlots is a major issue in the field establishment of the crop, resulting in poor emergence and uneven establishment. *E. angustifolia* seeds have a non-deep physiological dormancy (discussed in the previous chapter) with a physiological inhibiting mechanism of the embryo and/or covering structures (endosperm or testa). This dormancy can be successfully broken by a period of cold stratification in water or ethephon solution (Feghahati and Reese, 1994; Macchia *et al*., 2001, Cover, 2004). However, maintenance of seed quality during long periods of stratification in water or ethephon can be problematic for large seedlots. The requirement of cold temperature on imbibed seeds suggested that dormancy release can be achieved by sowing seeds in the colder months, without the need for pre-treatments. (Blake, 1935; Salac, 1982; Smith–Jochum and Albercht 1987, 1988; Parmenter *et al*., 1996).

Induced secondary dormancy is a possible reason for poor emergence of *E. angustifolia* in the field, however, there is no published literature regarding this issue. Parmenter *et al*., (1996) reported lowered germination in *E. angustifolia* possibly due to excessive drying and heating of seed trays under glasshouse conditions. However the literature did not indicate the viability of ungerminated seeds.

In the present experiment, the effects of field conditions on emergence were investigated at two sites with different climatic conditions and soil types. The

experiment was continued beyond the germination stage to investigate possible differences in the development of the seedlings through to flowering stage. The experiment was designed as a time of planting by pretreatments factorial intended to provide practical information on optimum sowing time and location for field production in Tasmania, as well as providing information on the possible induction of secondary dormancy in a field situation.

5.2: Materials and methods

5.2.1: Seedlot details

SL2003 seeds were used in this experiment due to lack of sufficient quantities of the more dormant SL2002. Untreated seeds were dry-stored at 4 ºC in plastic ziplock bags for the duration of the trial and germination and viability of seeds were checked before stratification pretreatments commenced.

5.2.2: Field sites

The experiment was conducted at two different sites in Tasmania, a property in Ulverstone (-41.1594S/146.1684E) and a second property at Glenora in the Derwent Valley (lat/long:-42.7117S/146.8975E). The two sites were chosen because the differing seasonal temperature conditions and soil types are representative of the range of the growing conditions likely to be experienced in Tasmania. Historical temperature records for the nearest weather stations to both sites, Bushy Park and Forthside Vegetable Research Station, are given in Table 5.2.1. The soil types were red ferrosol in Ulverstone and alluvial sandy loam (river flat) at Glenora.

Table 5.2.1: Long term mean monthly maximum and minimum temperatures for the Bushy Park Estates (Site no. 095003, Latitude:-42.7117 S, Longitude: 146.8975 E, Elevation: 35.0m) and Forthside Vegetable Research Station (Site no. 091186, Latitude:-41.2053 S, Longitude: 146.2631 E, Elevation: 130.0m) weather stations. Both trial sites are within 10 km of the nearest weather stations. (Only the months of the experimental trial are shown). Source: Australian Bureau of Meteorology.

Month	Maximum temperatures (^{o}C)		Minimum temperatures(^{o}C)	
	Bushy Park	Forthside	Bushy Park	Forthside
May	14.2	14.4	4.1	6.2
June	11.2	12.2	2.2	4.1
July	11	11.5	1.5	3.6
August	13	12.1	2.4	4.2
September	15.4	13.3	3.9	4.9
October	17.6	15.4	5.8	6.2
November	19.7	17.1	7.5	8.1
December	21.9	18.9	9.1	9.6

5.2.3: Cultural details

Fertiliser and lime applications were applied consistent with current commercial practices for similar crops to supply adequate levels of nutrients and to increase the soil pH to within an acceptable range (7-8) for *E. angustifolia* (Foster, 1991). The experimental sites were sprayed with Roundup® (glyphosate isopropylamine salt) herbicide approximately two weeks prior to sowing. During the early stages of seedling establishment plots were hand-weeded as required. After establishment, Gesagard® (2,4-bis[isopropylamino]-6-[methylthio]-s-triazine) was used on the Ulverstone site for weed control whereas hand-weeding was used at Glenora. All trial plots were irrigated as required with overhead sprinklers, with application rates based on published Class A pan evaporation figures (Bureau of Meteorology).

5.2.4: Time of planting and seed pre-treatments

Field plantings were made at each site during the middle of May, July, September, October and November of 2005. For each planting date, four seed pre-treatments were compared with non-treated (control) seeds. Seed pre-treatments, identified in the literature (Feghahatti & Reece, 1994; Parmenter, 1992; Cover, 2004) and in Chapter 3 of the current study, as potential strategies for breaking dormancy in *Echinacea angustifolia*, were:

1. water stratified and re-dried (WD)
2. water stratified and sown wet (WW)
3. ethephon treated and re-dried (ED)
4. ethephon treated and sown wet (EW)

Seeds were soaked in 15 mL of deionised water in plastic containers with perforated lids for 4 weeks at 4 ^{o}C for the water treatments (1 & 2). The ethephon pre-treatments (3 & 4) were similar but with 15 mL of 10^{-3} M ethephon instead for 2 weeks. Each pretreatment was applied to four replicates of 500 seeds. Containers were checked weekly and germinated or mouldy seeds were removed. Following pre-treatment, the seeds were either blotted dry on two layers of paper towels and then placed in air-tight glass phials as is (non re-dried treatments), or dried in net bags on a fan-assisted dryer for the dry-back treatments (3.2.3 in Chapter 3). Re-dried seeds had a moisture content of approximately 30% (w:w). Seed pre-treatments were timed for completion immediately prior to each time of sowing. Seed germination capacity and viability were checked before and after pre-treatments for each sowing date. That is, replication occurred during pretreatment and there was no storage of pretreated seeds between sowing times.

The experiment was a factorial with the factors arranged in a split plot design. The main plots were time of sowing set out as a randomised complete block, and the subplots were seed pre-treatments. Each subplot was a 3.5 m (Ulverstone) or 5 m (Glenora) long section of raised bed, containing 4 rows of 100 seeds, with an inter row space of 200 mm, giving 400 seeds per plot. Approximate intra-row seed spacings were 35 mm at Ulverstone and 50 mm at Glenora. The reason for the differences in subplots and spacing was due to a smaller plot size at Ulverstone. Both trials were hand sown, with seeds buried to a depth of ~12 mm. A single seedling tray with a plastic net lining was buried at soil level at one end of each sub-plot, across two of the rows and over sown with 20 seeds. This allowed for retrieval of ungerminated seeds for viability testing at the completion of the trial. At both sites, Tinytag® dataloggers mounted in Stevenson screens were used to log hourly air temperatures one metre above ground level. Hobo-Temp® dataloggers were buried at approximately the same depth as the seeds to record hourly soil temperatures during the trial.

Emergence data was collected from a randomly selected 500mm section of each of the four rows in the subplot. The first 300 mm of the row was disregarded to avoid inconsistencies in sowing techniques. The first germination counts were done four weeks after sowing, and then every two weeks at Ulverstone and monthly at Glenora. Germination at each time was expressed as a percentage of the total number of seeds sown, with germination assumed to be complete when no further changes in percentages were recorded.

At the end of the trial, sample plants were taken for assessment of stage of growth and dry weight. Ten samples (except where there was insufficient number of plants) were removed one metre from the end of each of the four rows of each replicate. The samples were dried at 50 ^{o}C for five days (until constant weight was achieved) and the dry weight recorded.

5.2.5 Statistical Analysis

Final (total) germination results were analysed using ANOVA (SPSS general linear models package). Percentage results were tested for normality and no transformations were carried out prior to analysis. Means were compared using Fischer's least significant difference (Steele and Torrie, 1981) at P=0.05, where significant (P<0.05) treatment or planting time effects, or the interaction, occurred. Germination profiles were plotted using mean and standard error figures for each pretreatment and planting time combination, but no statistical comparison between profiles was attempted. The two sites were analysed separately for all results.

5.3: Results

5.3.1: Initial seedlot viability and germination characteristics

The SL2003 seedlot used in this work had an initial germination capacity (GC) of 73.56% and an initial viability of 89.96%. The difference between these figures (16.40%) was taken to represent the percentage of seed with primary dormancy within the seed lot. The germination profile showed a steep peak at day 3 (Fig. 5.3.1).

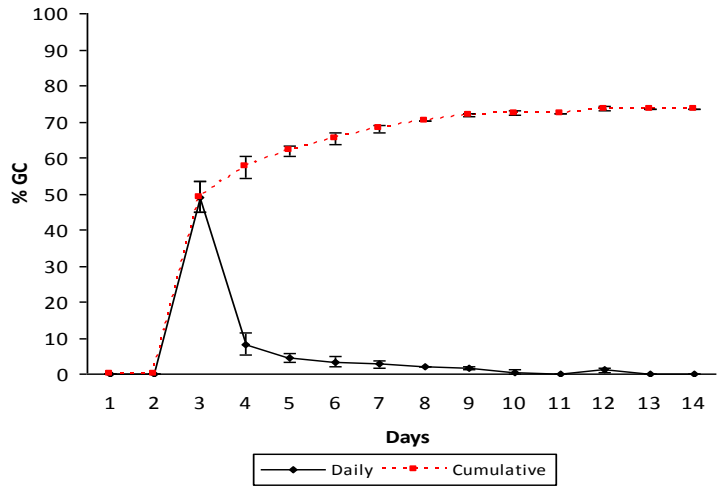


Figure 5.3.1: Daily and cumulative germination profile of *E. angustifolia* SL2003. Each data point is a mean of 4 replicates.

5.3.2: Effect of pre-treatment and storage on *in-vitro* seedlot viability and germination characteristics

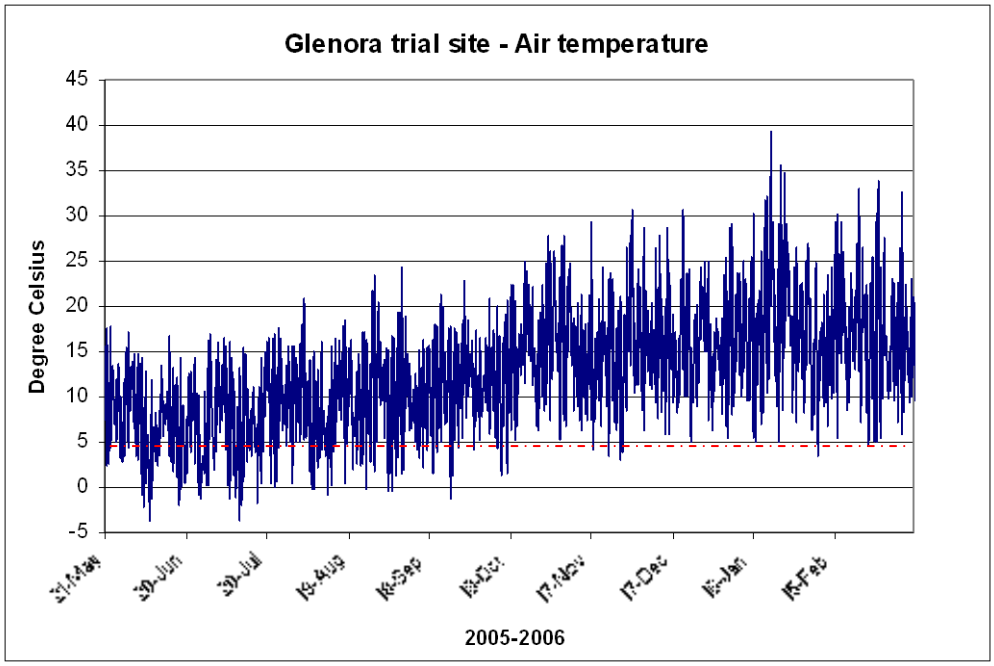
Throughout the experiment, stock seed viability was consistently above 80% with a mean of 86.8%, demonstrating no significant loss of viability over the seven months of storage covering the duration of time of sowing treatments. There was no evidence of a decline in germination capacity over the duration of the trial, with *in-vitro* controls having a mean GC of 65.7%, and pre-treated seeds consistently germinated above 80% (across all treatments) with a mean GC of 86.0% (Table 5.3.2). Control treatment germination was significantly ($P<0.05$) lower at all times, but there were no significant differences between treatments within or between times. Results of squash/TZ testing indicated very low level of primary dormancy (<2.7%) after pre-treatments.

Table 5.3.1: Post-treatment i*n-vitro* percentage germination capacity of *E. angustifolia* SL2003 over seven months.

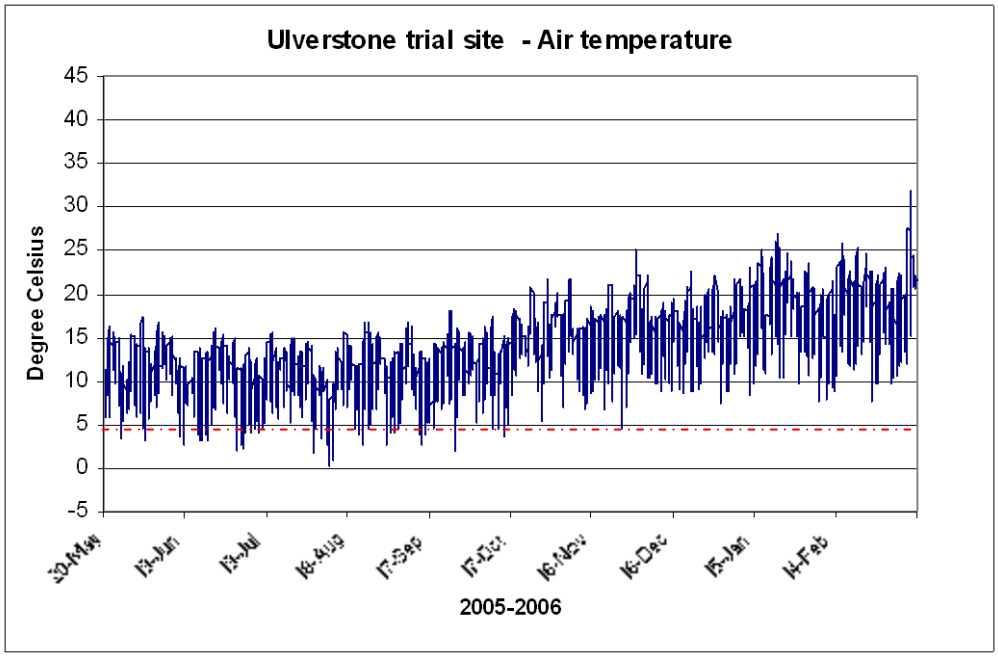
	Post-treatment GC %				
Treatment	May	July	Sept	Oct	Nov
Control	69.4	64.6	67.8	64.3	62.4
WD	90.9	85.1	85.0	84.8	84.6
WW	90.3	87.4	86.5	83.6	82.0
ED	91.1	87.6	82.7	86.3	86.3
EW	88.0	84.1	85.9	85.6	81.9

5.3.3: Effect of time of planting and pre-treatment on field emergence

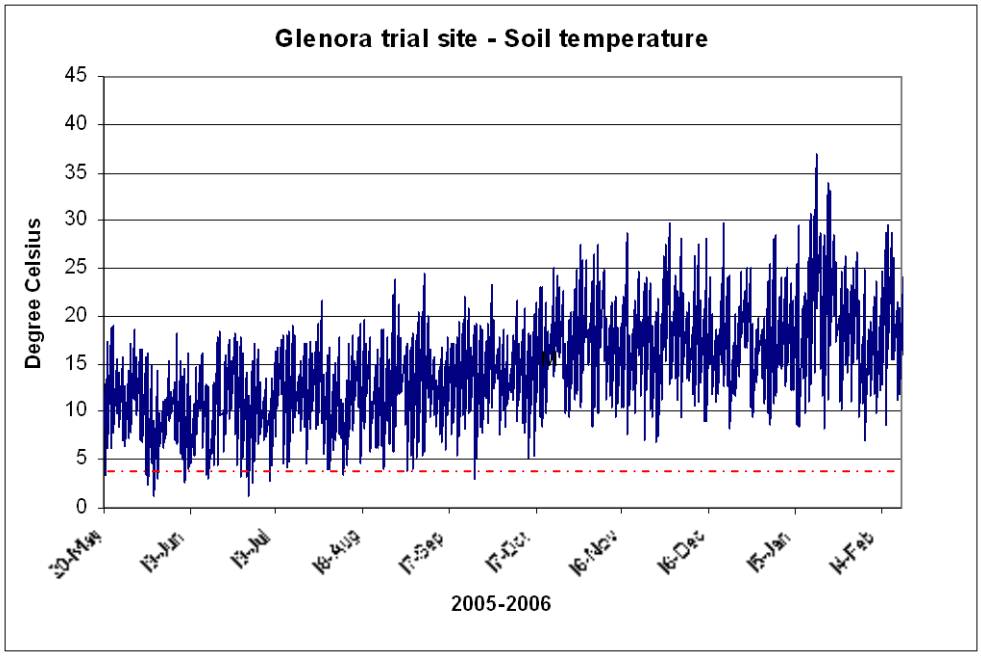
Air and soil temperature data for the two sites are shown in Figure 5.3.2(A-D). In general, air temperature conditions during the trial period were typical for each site (Figures 5.3.2 A&B) when compared with historical data at the nearest stations (Table 5.2.1). The Glenora site experienced cooler wintertime and warmer summertime conditions and greater daily extremes of temperature than the Ulverstone site which did not record any temperatures below 0 ^{o}C. Soil temperatures (Fig. 5.3.2 C&D) followed a similar pattern, although the differences between sites were smaller.


Glenora trial site - Air temperature
Degree Celsius
45
40
35
30
25
20
15
10
5
0
-5
2005-2006

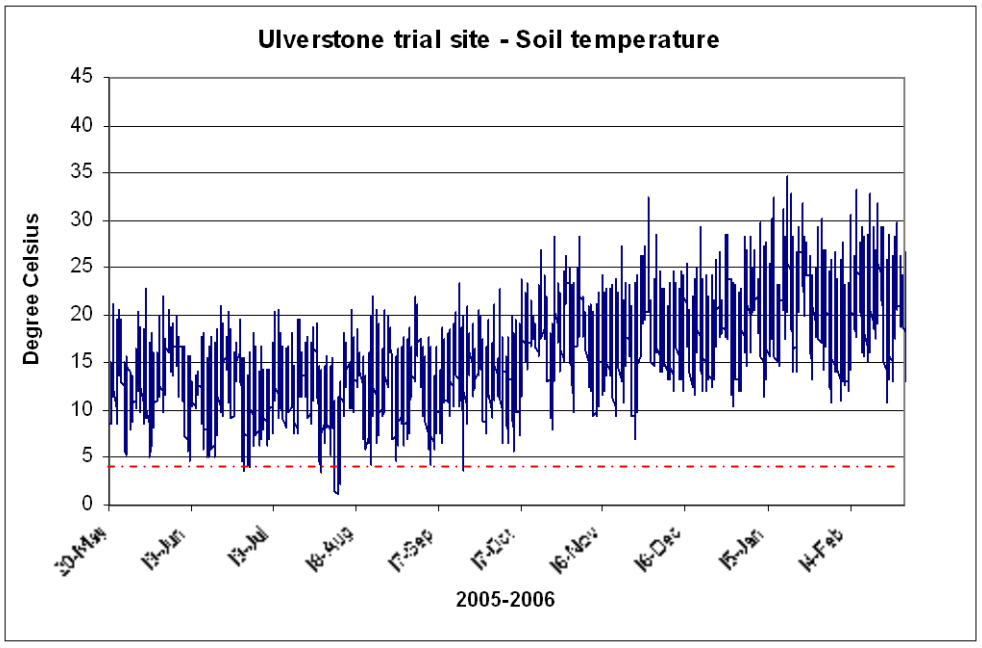
A


Ulverstone trial site - Air temperature
Degree Celsius
45
40
35
30
25
20
15
10
5
0
-5
2005-2006

B



C



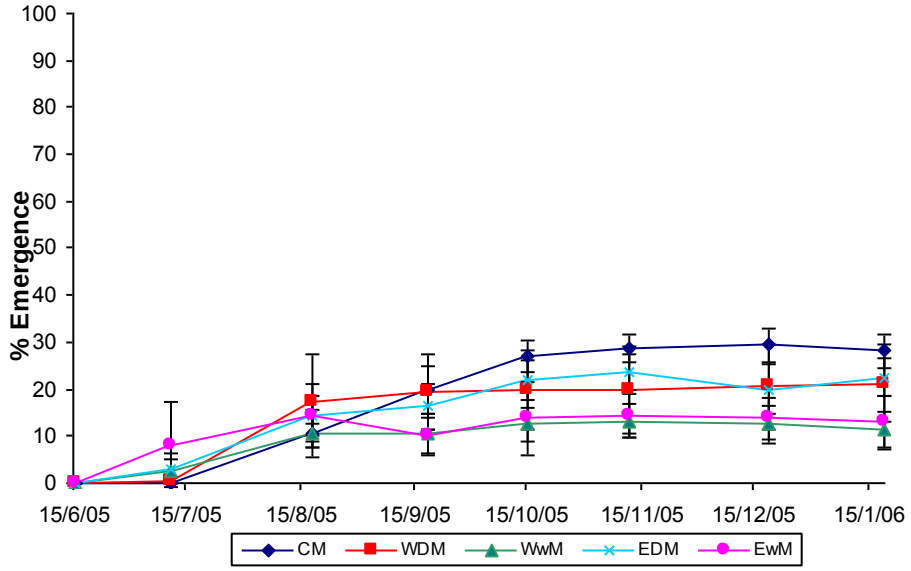
D

Figure 5.3.2: Air and soil temperatures data for duration of trial at Glenora (A & C) and Ulverstone (B & D). Red dash line indicates 4 ^{o}C which is the optimum temperature for dormancy release in *E. angustifolia* (*in vitro*).

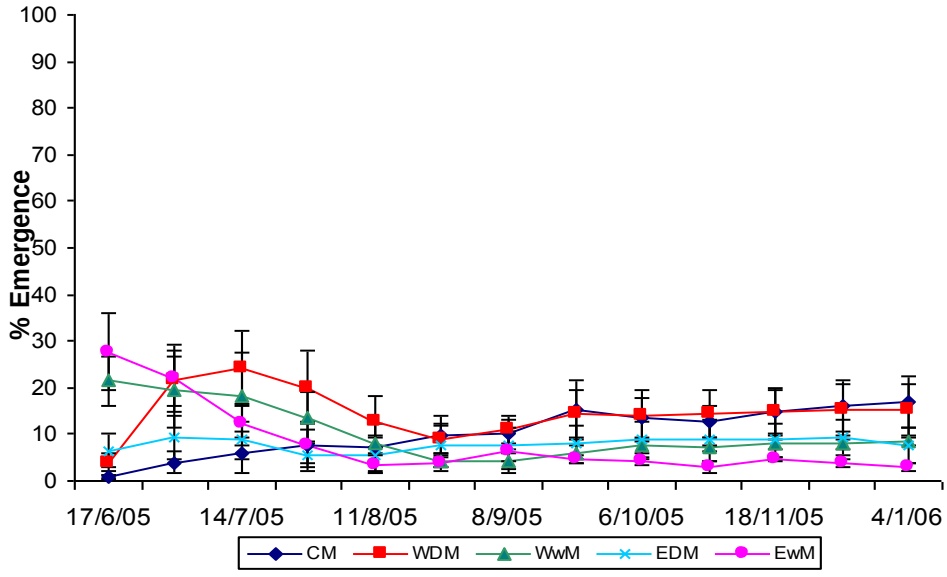
5.3.4 Effects on final germination

Overall for the two sites, there were significant ($P<0.001$) interactions between pretreatment and time of planting. There was also significant pretreatment ($P=0.022$) and month ($P<0.001$) effects at Ulverstone, but not at Glenora.

Less than 30% of seeds from any pre-treatment planted in May resulted in established plants eight months after sowing at either field site (Fig.5.3.3). There was a significant ($P<0.001$) interaction between treatment and month and also a significant treatment ($P=0.022$) and month ($P<0.001$) effect for Ulverstone. Glenora had a similar interaction but treatment and month were both highly significant ($P<0.001$).



A



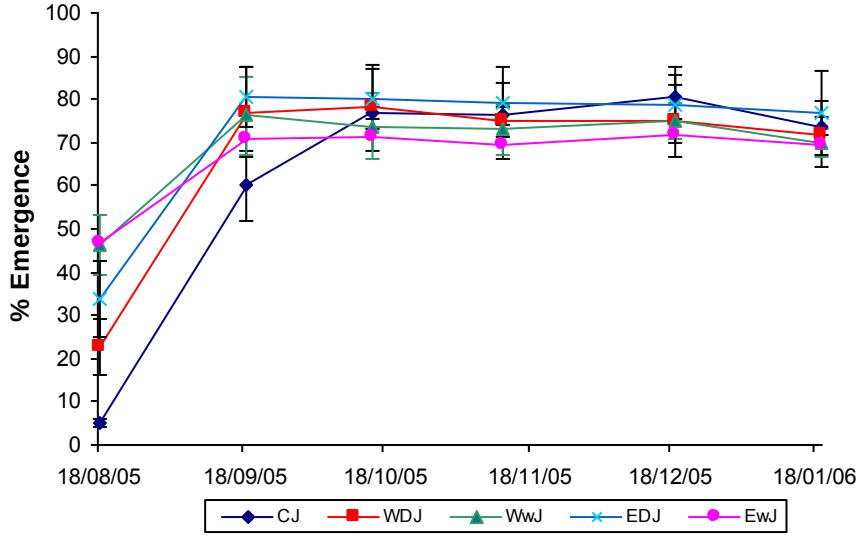
B

Figure 5.3.3: Emergence profile over eight months in 2005-6 - May sowings at Glenora (A) and Ulverstone (B). Legend: C=control, WD=water-stratified (re-dried), Ww=water-stratified (not re-dried), ED=ethephon-stratified (re-dried), Ew=ethephon (not re-dried) and M=sown in May.

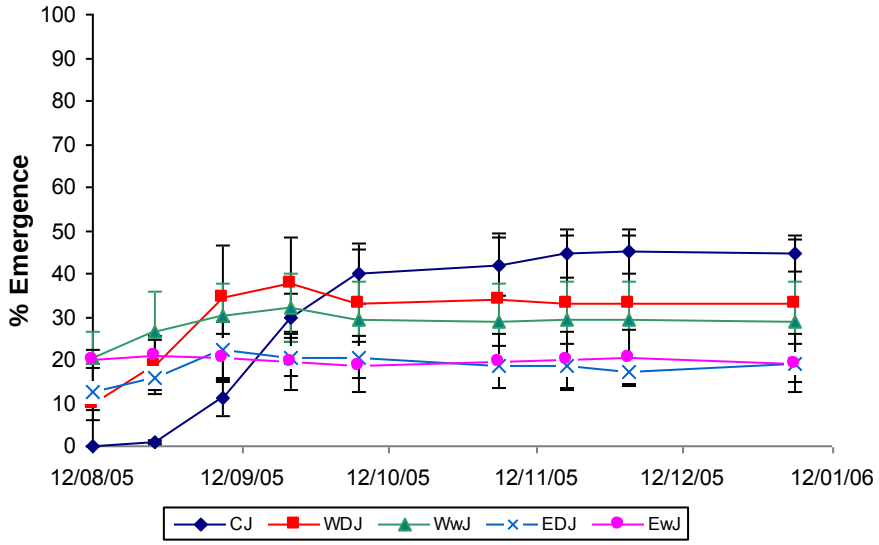
Maximum establishment of untreated (control) seed at both sites was recorded for July sown seed, with greater than 75% emergence at Glenora and approximately 45% emergence at Ulverstone. Within the July sowing, treated seeds emerged earlier and over a shorter period but the control seeds had attained similar (Glenora) or higher (Ulverstone) emergence by October, 95 days after sowing (Fig.5.3.4).

Spring sowings (September to November) showed a different emergence profile from late autumn and winter sowings, with pretreated seeds showing a synchronised emergence with little or no further emergence after the first assessment. A progressive decline in maximum emergence of the control (non-treated) seeds was observed at both sites, with control seeds only attaining a maximum of 32.5% germination for the September sowing at Glenora, with even lower percentages (<20%) for October and November sowings (Figs. 5.3.5-7).

In contrast to the responses observed for non-treated seeds, there was a marked increase in rate of emergence of pretreated seeds sown in spring, compared to July at both sites, and an improvement in total emergence from later sowings between July and October at Ulverstone (up to 74.0% emergence). Except for the month of October, when emergence was similar at both sites, Glenora was consistently higher with an overall emergence and establishment of 48.9% compared to 31.0% at Ulverstone (Fig. 5.3.8A&B). These figures were well below the *in vitro* overall germination of 81.7% (Fig. 5.3.8C).

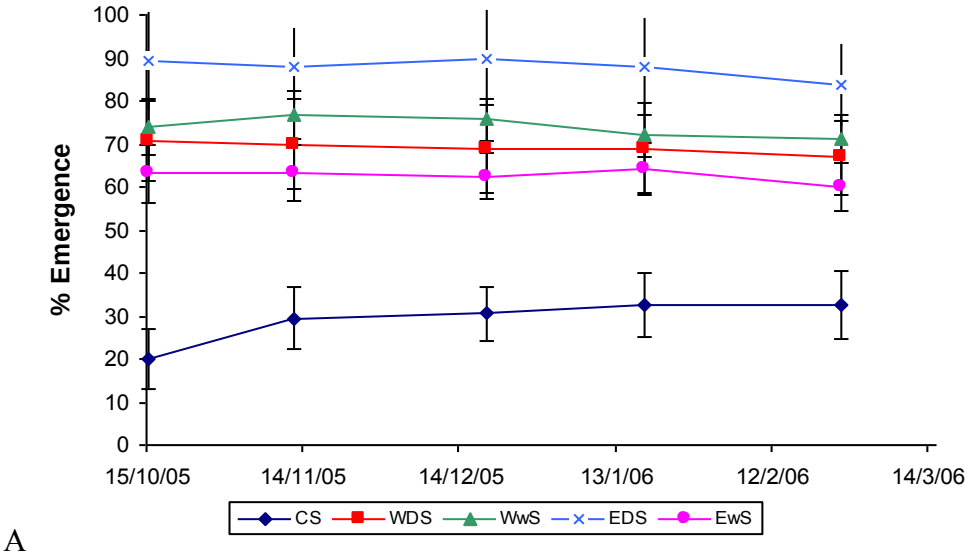


A

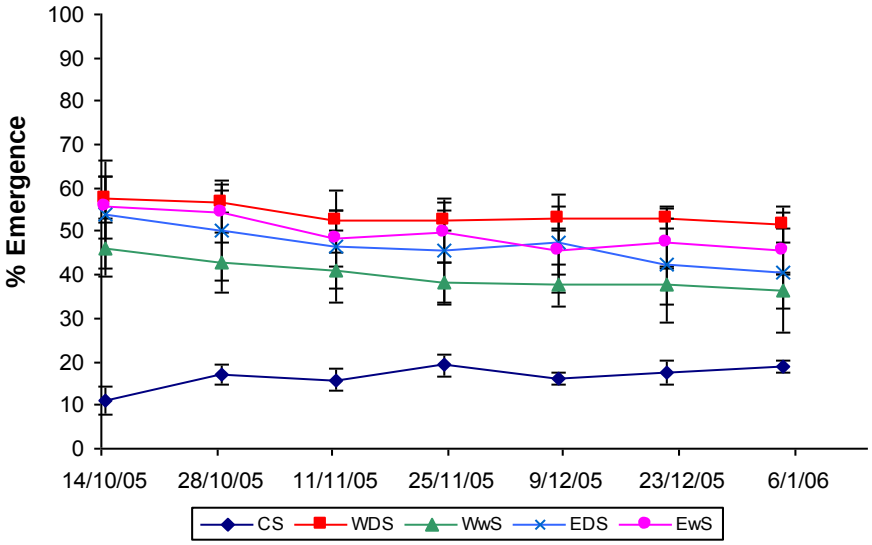


B

Figure 5.3.4: Emergence profile of *E. angustifolia* SL2003 from July 2005 sowing, Glenora (A) and Ulverstone (B). Legend: C=control, WD=water-stratified (re-dried), Ww=water-stratified (not re-dried), ED=ethephon-stratified (re-dried), Ew=ethephon (not re-dried) and J=sown in July.



A



B

Figure 5.3.5: Emergence profile from September sowing, Glenora (A) and Ulverstone (B). Legend: C=control, WD=water-stratified (re-dried), Ww=water-stratified (not re-dried), ED=ethephon-stratified (re-dried), Ew=ethephon (not re-dried) and S=September sown.

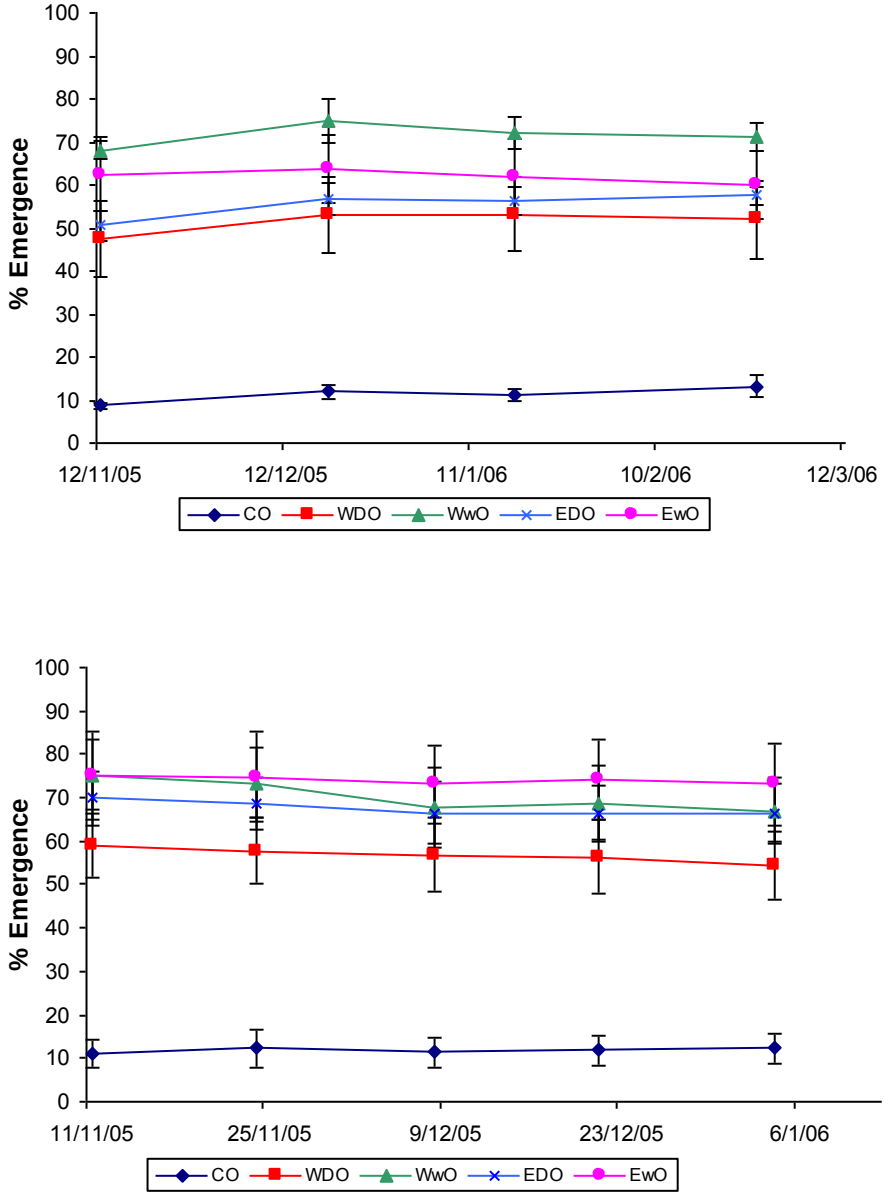


Figure 5.3.6: Emergence profile from October sowing, Glenora (A) and Ulverstone (B). Legend: C=control, WD=water-stratified (re-dried), Ww=water-stratified (not re-dried), ED=ethephon-stratified (re-dried), Ew=ethephon (not re-dried) and O=October sown.

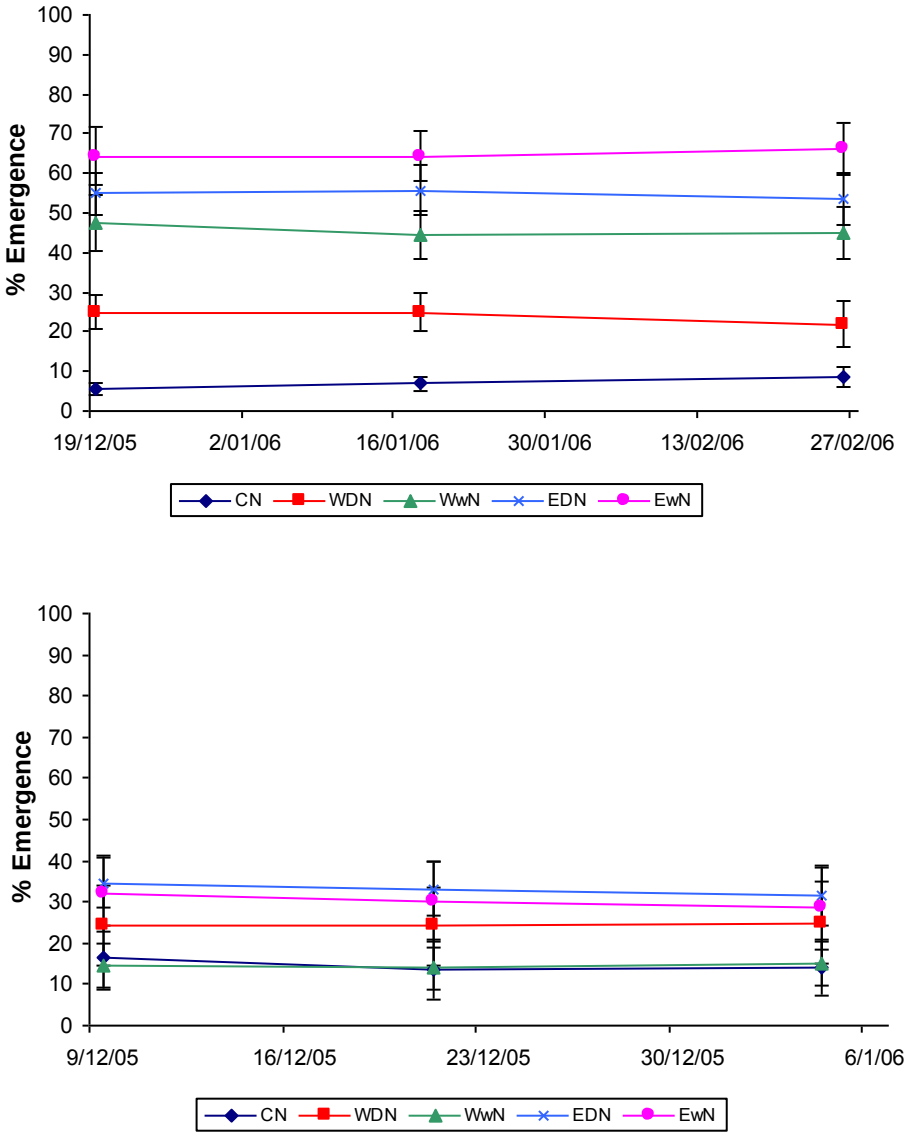
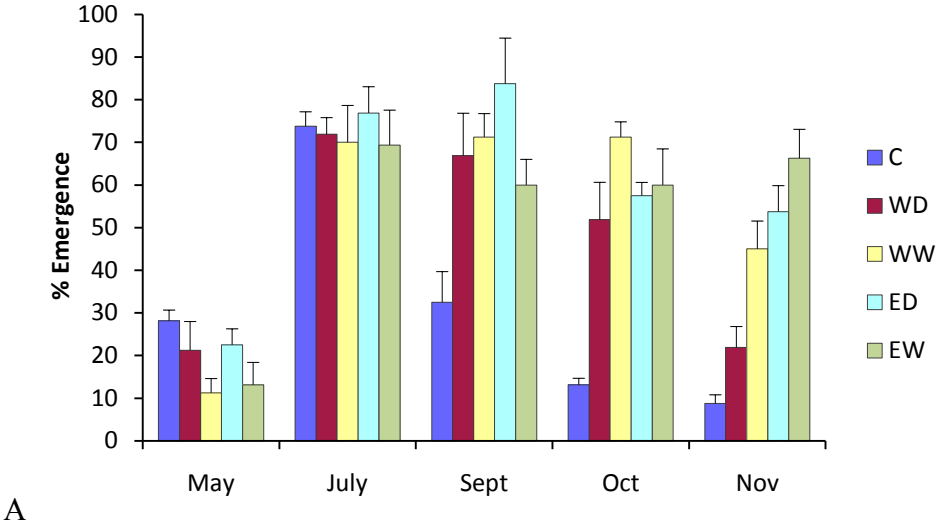
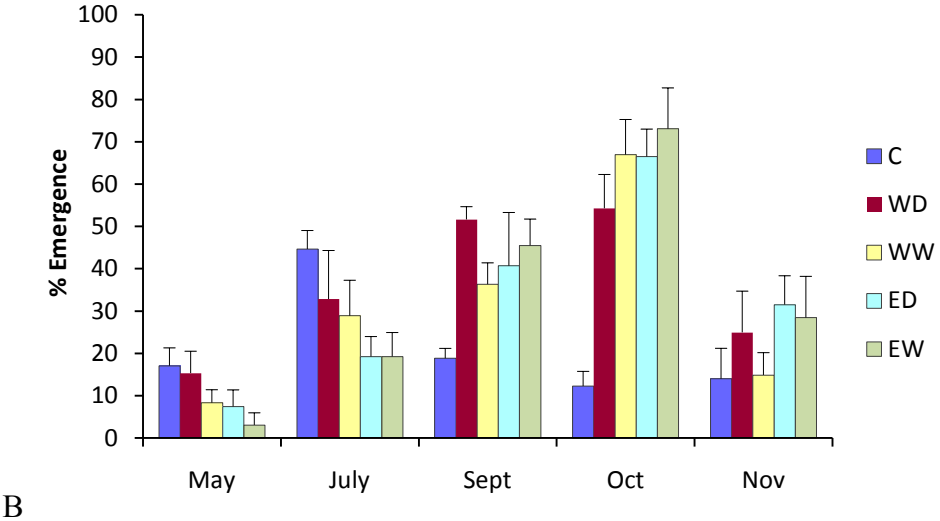


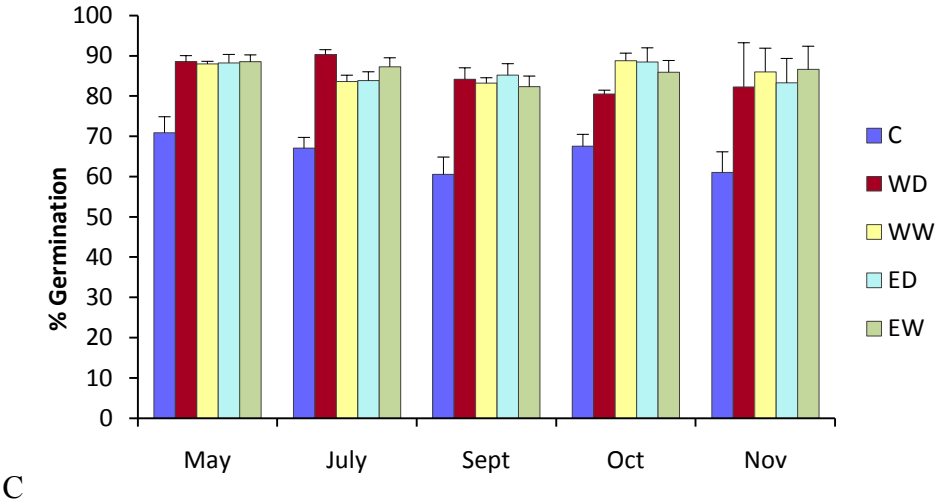
Figure 5.3.7: Emergence profile from November sowing, Glenora (A) and Ulverstone (B). Legend: C=control, WD=water-stratified (re-dried), Ww=water-stratified (not re-dried), ED=ethephon-stratified (re-dried), Ew=ethephon (not re-dried) and N=November sown.



A



B



C

Figure 5.3.8: Overall emergence of *E. angustifolia* SL2003 at Glenora (A) and Ulverstone (B) compared to *in vitro* germination (C) for the same time period.

5.3.4: Viability of ungerminated seeds

Ungerminated seeds, retrieved from buried trays ten months after sowing, from the May and July sowings at both sites, were almost entirely non-viable with less than 1.25% viability (Fig 5.3.9A&B). However, there were a higher percentage of viable seeds retrieved from the November sowings (all treatments) and from the September and October controls. Emergence in the trays was generally lower, but conformed to the emergence trend from the rest of the plots except for the month of November (Fig. 5.3.10). There was a high percentage (48.8%) of viable, non-germinated seeds for the water-stratified, redried seeds for November at Glenora which was inconsistent with the other treatments.

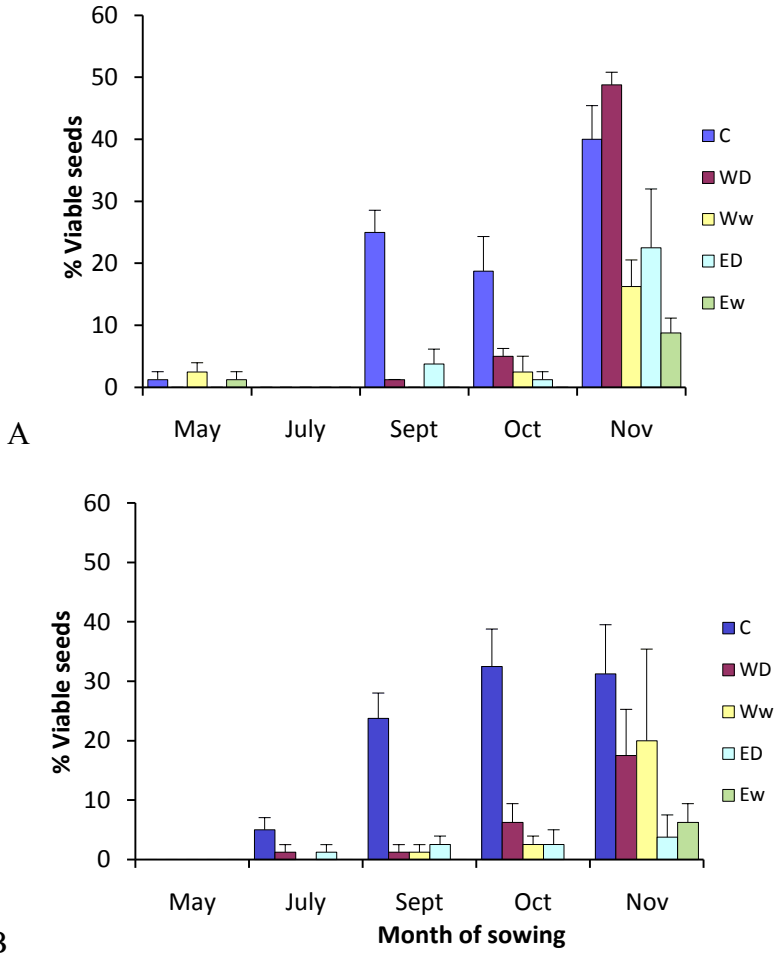
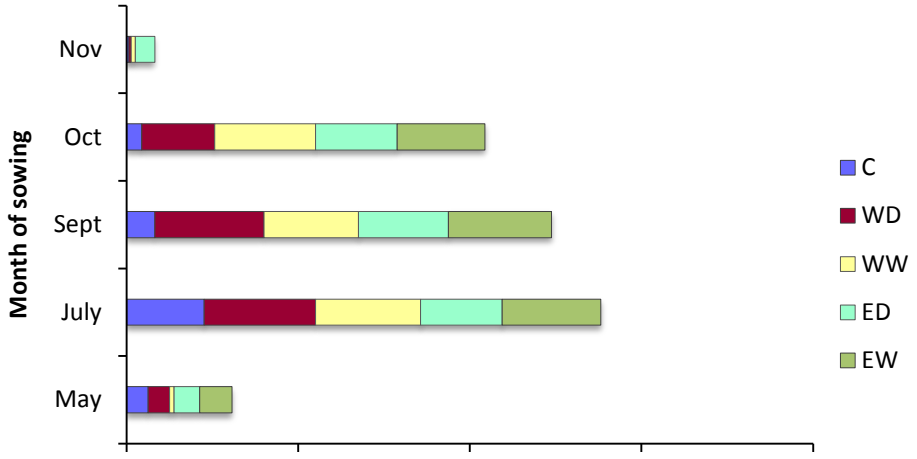
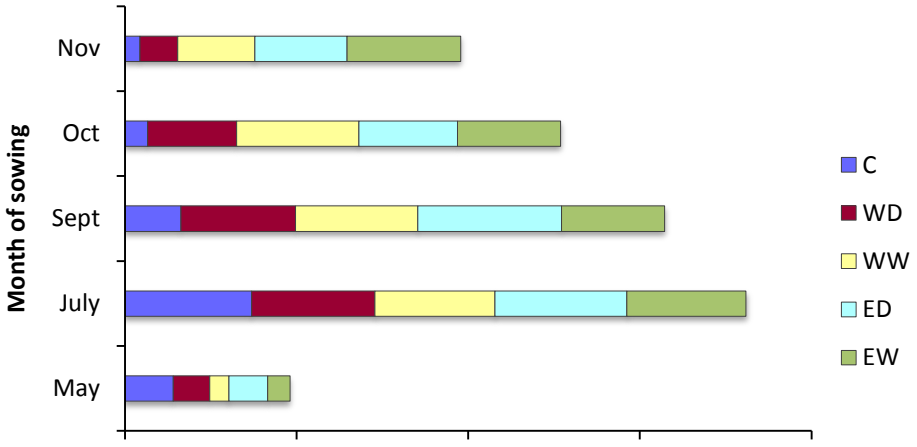


Figure 5.3.9: Percentage of viable seeds of *E. angustifolia* SL2003 retrieved from buried trays at the end of the trial at Glenora (A) and Ulverstone (B).



A



B

Figure 5.3.10: Comparing the trend of emergence of *E. angustifolia* SL2003 in the buried trays (A) with the rest of the trial plots (B) at Glenora.

5.3.5: Comparison of growth stage between sites

There were marked differences between the two sites in stage of growth at the end of the experiment. At Ulverstone, many of the plants had achieved anthesis, and although a few individuals at Glenora had also reached that stage or were developing reproductive

buds, most were still in a vegetative stage of growth (Figs.5.3.12-13). Dry weights are shown in Table 5.3. There were also large variations in plant growth stages even within the same replicate (Figs. 5.3.14-15) but uniformity was more evident at Ulverstone. Some individuals were taxonomically closer to *E. purpurea* with fibrous roots and slightly broader leaves (Fig. 5.3.11A) compared with the typical long tap rooted *E. angustifolia* (Fig 5.3.11B).

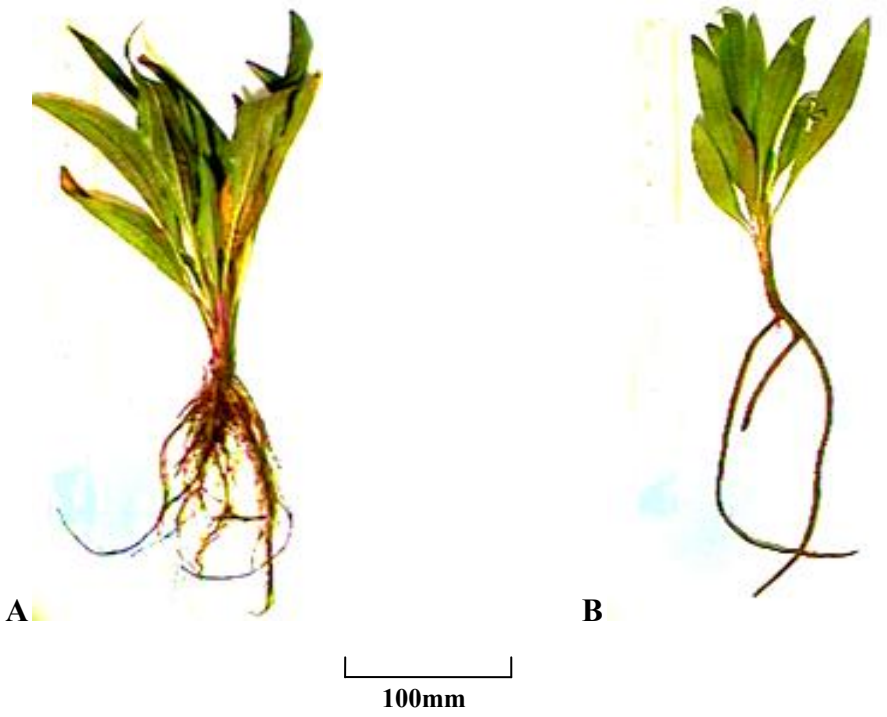


Figure 5.3.11: Two different forms of *E. angustifolia* SL2003 plant samples collected from field experiment at Ulverstone. Sample B has the typical long tap roots of the species.

Figure 5.3.12: Best plot of *E. angustifolia* SL2003 at Ulverstone with 75% emergence, October sowing – treatment: water stratified & sown wet. Photograph taken in March 2006

Figure 5.3.13: Best plot of *E. angustifolia* SL2003 at Glenora with 90.0% emergence, September sown – treatment: ethephon dried back. Photograph taken in March 2006.

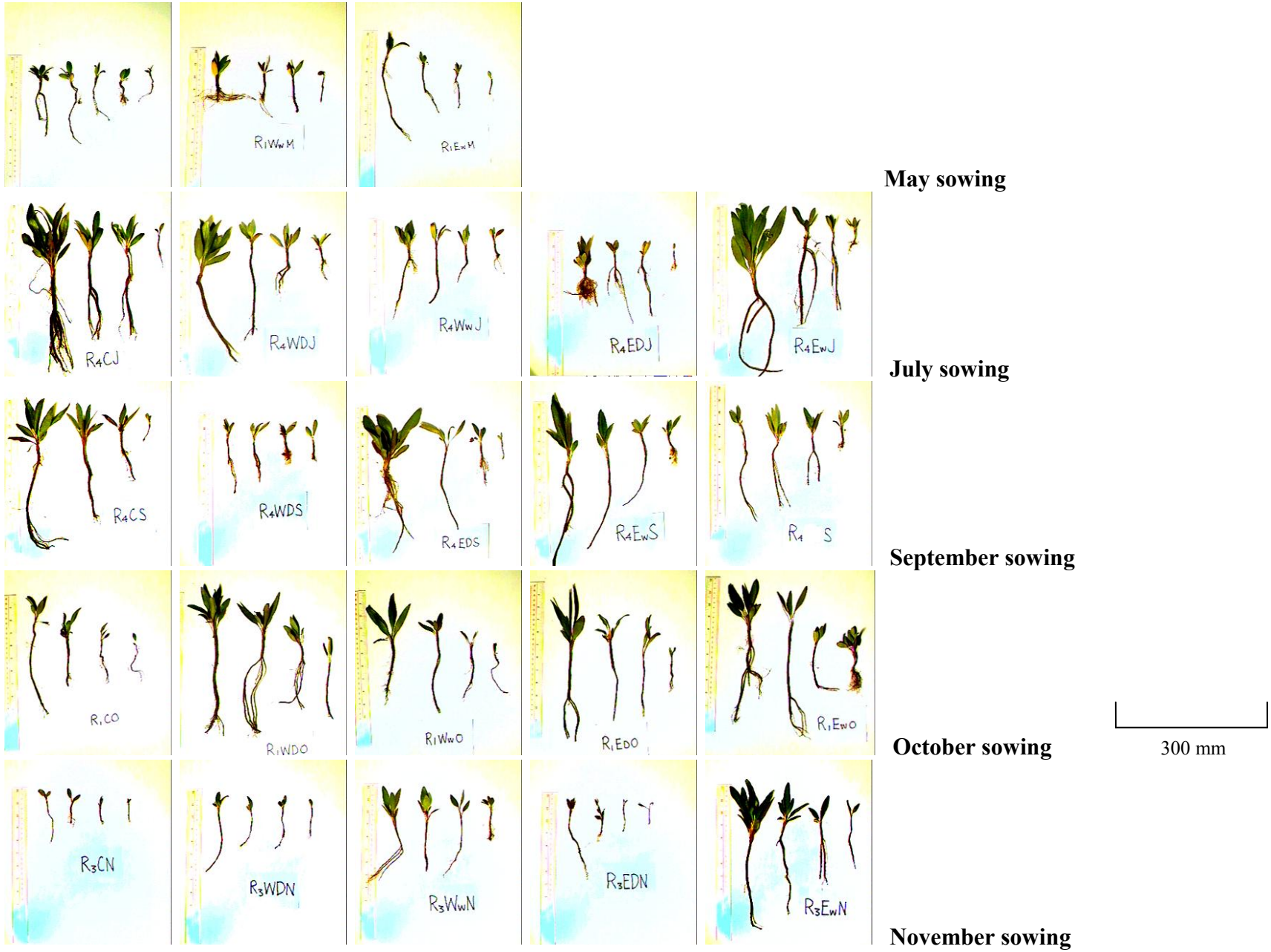


Figure 5.3.14*: E. angustifolia* SL2003 samples taken from Glenora on 5/4/2006 at the end of trial. Legend: R= replicate, M=May, J=July, S=Sept, O=Oct, N=Nov; C=control, WD=water-stratified & redried, Ww= water-stratified sown wet, ED=ethephon-stratified & redried, Ew=ethephon-stratified, sown wet.

Figure 5.3.15: *E. angustifolia* samples taken from Ulverstone on 28/3/2006 at the end of trial showing variability within and between replicates
Legend: R= replicate, M=May, J=July, S=Sept, O=Oct, N=Nov; C=control, WD=water-stratified & redried, Ww= water-stratified sown wet, ED=ethephon-stratified & redried, Ew=ethephon-stratified, sown wet.

Table 5.3.2: Dry weight of *E. angustifolia* plant samples for different seed pretreatments (C=control, WD=water-stratified & redried, WW=water-stratified & sown wet, ED=ethephon-stratified & redried, EW=ethephon-stratified & sown wet) from five sowing dates at two sites – Glenora (G) and Ulverstone (U). Each figure (in mg) represented the mean of 40 plants from four replicates except where indicated by subscripts. Blank cells referred to unavailable samples.

	Seed Pretreatments									
	C		WD		WW		ED		EW	
	G	U	G	U	G	U	G	U	G	U
May	77.50_{36}	395.43	104.41_{36}	294.10	98.09_{30}		40.44_{31}		50.43_{34}	
July	99.56_{34}	408.83	67.38_{34}	403.92_{36}	90.74_{34}	417.02_{34}	37.21_{31}	337.80	54.55_{36}	268.84_{34}
September	91.25_{38}	288.48	86.65_{36}	360.01_{38}	57.35_{36}	449.15	94.08_{37}	314.18_{37}	83.95_{38}	379.58
October	40.47_{28}	163.05	49.20_{33}	205.15_{26}	48.54_{34}	219.93	46.5935	223.75	50.93	247.83
November	18.91_{28}	55.83_{29}	17.61_{26}	93.88	25.51_{31}		12.31_{30}			

5.4: Discussion

Overall, all of the seed pre-treatments were effective in promoting germination under field conditions that were unfavourable for germination of non-treated seed in September and October (Ulverstone) and September to November (Glenora). Whilst significant variation in emergence between pre-treatments was observed on individual planting dates, there did not appear to be a consistent difference between sites or between planting dates. Importantly, drying back after pre-treatment did not have a negative impact on promotion of germination. This has important implications for use of pre-treated seed in commercial sowings.

Based on seed lots used in previous work (Cover, 2004) and anecdotal evidence from commercial trials, the seedlot SL2003 used in this work had a relatively low level of primary dormancy. Observations of plant morphological variation in the established trial plots, ranging from plants that resembled *E. purpurea* to *E. pallida*, suggested that the low level of primary dormancy may have in part resulted from cross pollination of the stock seed parent line *E. angustifolia* with *E. purpurea* and *E. pallida.* Figure 5.3.1 showed a peak germination of 48.1% on Day 3 (compared to a final cumulative mean of 49.2%) which was atypical of a dormant seedlot such as SL2002 (Chapter 3, Fig. 3.1). Under these circumstances, a low emergence compared to *in vitro* results would indicate an unfavourable germination environment rather than issues related to primary dormancy.

From the temperature figures, Glenora presented more days with minimum air temperature below 4 ^{o}C with some sub-zero days between May and early September.

This is also reflected in soil temperatures although these were more moderate. For July sown seeds at Glenora, there is some evidence of natural release from primary dormancy (75% emergence compared to 67% germination capacity prior to sowing). This may explain the improved emergence in spring for untreated seeds at this site for the July sowings. This data agreed with Salac *et al*., (1982) who found 75% of *E. angustifolia* sown in November (northern hemisphere) germinated the following spring, compared to 5% sown after April. The more rapid emergence of treated seed in the July sowings compared with non-treated seed may reflect the fact that treated seed commenced the process of germination prior to planting; that is, that the treatments have had a priming effect. Although the May-sown seeds would have been subjected to the same (stratification) temperatures, the length of time in the ground may have resulted in deterioration and/or predation which were expressed in the very low (1.25%) viability in the recovered trays. Stratifying temperatures below 4^{o}C progressively reduced germination percentage with time (Chapter 4, Fig 4.5.1). Emergence of pretreated seeds from spring sowings paralleled *in vitro* results with synchronised emergence and almost no further emergence after the initial count. Although percentages were lower than *in vitro* data in all treatments, overall emergence of untreated seeds was 74.9% less in the field. This was reflected in the results from the buried trays.

The lower emergence percentages in the trays compared to the rest of the plot (Fig. 5.3.10) may have been due to their position at the end of row or a different microclimate had been created by the tray itself. Taking this into consideration, the data for viable seeds in the trays should also be correspondingly lower. However, the data still indicated that these seeds had re-entered a dormant state. The high percentage of viable

seeds for the spring sowing was expected as the period of interment is shorter and natural losses due to deterioration and predation are reduced. The lower percentage of viable seeds in the pre-treated seeds (for the months other then November) may be due to their reduced ability to withstand unfavourable environmental conditions after treatments. Given that 48.8% of the viable pre-treated seeds retrieved from the November trays (Figs 5.3.9) were higher than the level (16.4%) of primary dormancy initially observed in the seed lot, it appeared that the seeds may have entered secondary dormancy. Conditions favouring induction of secondary dormancy, therefore, had increased at later sowing dates. Potential factors may include temperature, soil water potential or photoperiod.

A decline in the number of seedlings over winter from most treatments in the May emergence at Ulverstone (Fig. 5.3.3B) suggested possible cold sensitivity or general attrition (invertebrate damage, etc.) during a period of extremely slow growth. However, this pattern was not repeated at the Glenora site (Fig. 5.3.3A) where frost events were common. A possible explanation may be that the temperatures may have been too cold at Glenora for the pretreated seeds to germinate as indicated by the first assessment and therefore did not show a decline in the number of seedlings over the winter months. Average soil temperatures were about 7 ^{o}C compared to around 14 ^{o}C for Ulverstone between mid May to mid August, with some days with air temperatures below 0 ^{o}C (Fig. 5.3.2).

The data collected from this experiment suggest that one of the key factors in poor establishment of commercial plantings in spring sowings could be due to the induction of secondary dormancy after sowing. The full potential for natural primary dormancy

release under field conditions remains unclear due to the low level of primary dormancy in the seedlot that was used. But given the low level of primary dormancy in treated seeds prior to sowing, there was further evidence of induction of secondary dormancy in spring sowings.

From observations of stage of plant growth and the dry weight data from the two sites, Ulverstone appears to have conditions more conducive to growth but emergence was generally lower than Glenora. The possibility of soil water potential may be worth investigating given the two distinctly different soil types.

5.5: Summary

At this stage, data is only available for one season of trials, with a seedlot of questionable heritage and low primary dormancy so any conclusions are tentative.

The results indicated that field conditions in May 2005 were not conducive for germination or seedling growth. Seeds remaining in the ground through winter lost their viability by the time conditions improved in September/October. July sowings were optimal for establishment of non-treated seed, although it is not clear why emergence at Ulverstone was much lower by comparison to Glenora.

Spring sowings required seed pre-treatment to ensure a reasonable emergence as temperatures did not appear to be low enough for dormancy release. Later spring sowings (November) may have resulted in induced secondary dormancy, even in pre-treated seed.

In general, the results from this trial show significant promise for field establishment of *Echinacea* under Tasmanian conditions. However they also raise a number of important research questions, particularly in relation to a possible induction of secondary dormancy. Clearly, there is considerable scope for further investigation of factors influencing field establishment.

Chapter 6: Investigating the possible induction of secondary dormancy

6.1: Introduction

Secondary dormancy is an induced or imposed state of dormancy in a mature dispersed seed as opposed to primary dormancy in a developing and maturing seed (Hilhorst, 1998). Environmental factors such as high or low temperatures, water stress, light or darkness, anaerobic conditions, gases such as ethylene and carbon dioxide and chemicals such as nitrates can induce secondary dormancy (Bewley and Black, 1982; Cadman *et al*., 2006). In nature, secondary dormancy as with primary dormancy is a survival mechanism to ensure germination in the most favourable conditions.

Ecological studies on buried seeds (Taylorson, 1970; Baskin and Baskin, 1985 & 1998) suggested that the seeds go through a „continuum' of dormancy/non-dormancy. This is a seasonal cycle of physiological changes during which the seeds demonstrate a widening of the temperature range for germination and vice versa (Figures 6.1.1 & 6.1.2). This cycling of dormancy appears to be a trait of seeds with non-deep physiological dormancy (Baskin and Baskin, 1998). Seeds may undergo induction and release of secondary dormancy during successive seasons until the right conditions become favourable (Hilhorst, 1998). Although classified separately, there are no published reports indicating a clear physiological or biochemical distinction between primary and secondary dormancy.

The mechanism involved in the induction of secondary dormancy is still unclear. Genotype is believed to be the principal controlling factor in, for example, western Canadian spring-sown *Brassica napus* (Gulden *et al*., 2004). Cadman *et al*., (2006) suggested that an abscisic acid/gibberellic acid hormone balance mechanism potentially controls the completion of germination; with dormant seeds having greatly reduced gene expression associated with protein synthesis which occurs during the germination process.

The membrane hypothesis (Hilhorst, 1998) proposed that membranes are the target of temperature perception and the resultant changes in the cellular (protein and lipid components) properties are involved in dormancy regulation. Anaesthetics such as chloroform and halothane are also believed to break dormancy by affecting the fluidity of membranes (Hallett and Bewley, 2002).

Aspects of respiration were also implicated in the induction of dormancy. Dormant seeds were hypothesised to be deficient in an alternative oxygen-requiring process (possibly the pentose phosphate pathway) essential for germination (Bewley *et al*., 2006). Respiration rates are also temperature-dependent, and there is often a proportional respiration-germination relationship (Dahal *et al*., 1996). There have also been suggestions of phytochrome involvement. Khan and Karssen (1980) induced dormancy in *Chenopodium bonus-henricus* L seeds with osmotic and high temperature treatments and the response could be prevented by light (far red followed by red) and growth regulator combinations. There were similar results with *Rumex crispus* L. reported by Samimy and Khan (1983), with a decrease in the ability of growth regulators to remove secondary dormancy induced by dark incubation with increasing

duration. All of these hypotheses, however, apply to both primary and secondary dormancy.

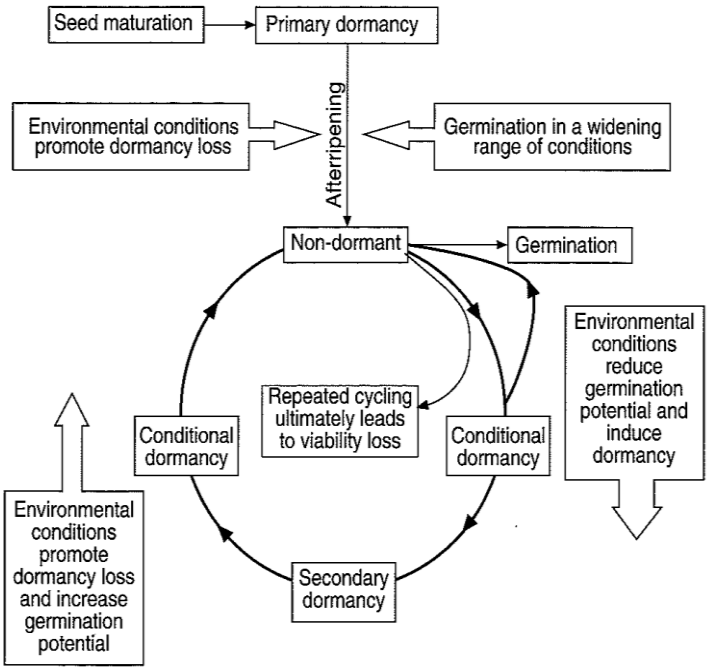


Figure 6.1.1: The change in status of a mature seed as it loses primary dormancy and enters a dormancy cycle. Once primary dormancy is lost, the non-dormant seed will germinate in a wide range of conditions. As secondary dormancy declines, the seed returns to a non-dormant state (Bewley *et al*., 2006, as adapted from Baskin & Baskin, 1998).

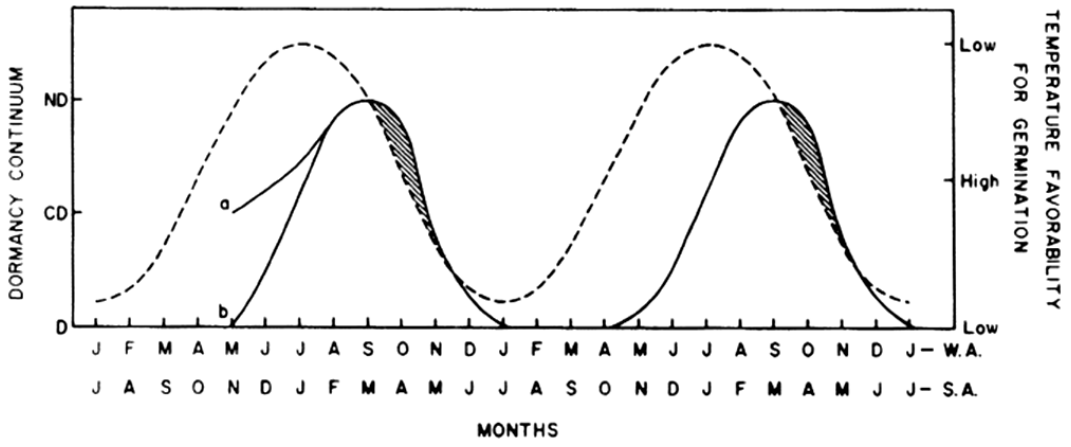


Figure 6.1.2: The annual dormancy cycle in buried weed seeds for strict winter annuals (W.A.) and spring-germinating summer annuals (S.A.). Hatched areas show when germination is possible. D = dormant, CD = conditionally dormant, ND = nondormant, a = seeds conditionally dormant at maturity, b = seeds dormant at maturity. Solid line = dormancy continuum, dotted line = temperature favourability for germination (Baskin & Baskin, 1985).

From the literature reviewed, unfavourable environmental factors are common initiators in the dormancy induction process. Much of the literature on secondary dormancy has come about from studies on weed seed germination, and the inducing factors are part of the natural climatic conditions. The main factors involved in the induction of secondary dormancy appear to be temperature and water stress (Bouwmeester and Karssen, 1989; Gulden *et al*., 2004; Mennan and Ngouajio, 2006).

6.1.1: Temperature

Temperature is the major factor that controls the induction of secondary dormancy although other factors may have prevented germination initially (Baskin & Baskin, 1998). Lettuce (*Lactuca sativa* L.) germination, known to be strongly temperature dependent, can be induced into secondary dormancy (commonly known as thermodormancy) in the range 25-30 ^{o}C (Kristie *et al*., 1981). Dormancy in *Amaranthus caudatus* was induced at 45 ^{o}C, but can be reversed by exposure to pure oxygen (Kepczyńki and Bihun, 2002). Apple seeds, following release from primary dormancy, were sensitive to induction into secondary dormancy at germination temperatures above 30 ^{o}C (Visser, 1954). Khan and Karssen (1980) found that darkness at 29 ^{o}C was a prerequisite for the induction of light-reversible secondary dormancy in imbibed seeds of *Chenopodium bonus-henricus*. Secondary dormancy can be induced in *Orobanche* spp. by decreasing temperatures from 20 ^{o}C to 10 ^{o}C or increasing to 30 ^{o}C (Kebreab and Murdoch, 1999). However, the authors did not mention whether the low germination could be due to reduced seed viability at high temperatures rather than the induction of secondary dormancy.

6.1.2: Water stress

The water potential (ψ_w) of a seed or other medium such as soil is the sum of four components: osmotic (ψ_π), pressure (ψ_p), matric (ψ_m) and gravitational (ψ_g) potentials (Bewley *et al*., 2006):

$$\psi_w = \psi_\pi + \psi_p + \psi_m + \psi_g$$

Gravitational potential is mainly disregarded, and initial water potential in dry seeds is mainly driven by the a matric potential which is between -35 and -50 MPa (in a dry seed). This drives water uptake in the imbibing seed during Phase I water uptake, but as ψ_m becomes zero, water entry into the seed decreases to almost nil (Phase II). Further water absorption (Phase III) is driven mainly by decreased osmotic potential in the cells resulting from mobilization of solute molecules (Bradford, 1995; Bewley *et al*., 2006). The ability of seeds to take up water and enable the radicle cells to maintain a turgor force which causes cell extension, elongation and radicle emergence, is said to be poor or lacking in dormant seeds (Bewley and Black, 1982). Esashi *et al*., (1983) found that the establishment of secondary dormancy was associated with a decrease in growth potential of axial and cotyledonary tissues in *Xanthium pennsylvanicum*.

Water availability and the rate of flow to the seed are dependent on the difference in water potential between the seed and the surrounding medium (Bewley and Black, 1994). Soil water potential is governed by the osmotic and matric potentials of the soil; the latter is due to capillary sorption of soil water into the soil matrix and is close to zero at saturation. Soil is not a uniform medium and the potential gradient (and hence uptake of water by seed) is also dependent on the seed-soil contact, as well as factors

such as the water-holding capacity and hydraulic conductivity of the soil, and capillary and vapour movement of water in the vicinity of the seed (Bewley and Black, 1982).

Water stress is not restricted to insufficient water in the soil but also to water-logging. For example, seeds of *Setaria parviflora* enter secondary dormancy in the soil during flooding (Mollard *et al*., 2007). Much of the literature on water-logging has focused only on impacts on germination and data on effects on secondary dormancy *per se* are not readily available. Measured non- or reduced germination may be due more to deleterious effects (soaking injuries and mortality) on the seeds than to induction of dormancy. Seed mortality is usually linked to alterations in respiratory metabolism including oxygen uptake (Roberts, 1969, 1973; Hilton and Thomas, 1987) and production and accumulation of toxic levels of acetaldehydes and ethanol (Woodstock and Taylorson, 1981). Secondary dormancy in *Echinochloa crus-galli*, due to oxygen deficiency associated with submergence was found to be temperature-related (Honěk and Martinková, 1992). Tolerance to water-logging appears to be species-specific (Urbieta *et al*., 2008; Pérez-Ramos and Marañón, 2009).

6.1.3: Secondary dormancy release

The release from secondary dormancy appears similar to primary dormancy and methods of removing the latter are also effective for the former. Cold stratification is successful in many species, including *E. angustifolia*, and hormonal treatments were successful in others.

6.1.4: Summary

Sown seeds are subject to changes in their immediate environment. Soil structure, water status and temperature will affect whether the seeds have the capacity to proceed to completion of germination or whether induction of secondary dormancy will occur. From the literature, temperature and water potential appeared to be the major parameters governing the induction of secondary dormancy.

Data from the previous chapter indicated a possible induction of secondary dormancy in the spring-sown seeds, especially in the November sowing. The most likely environmental factors appeared to be temperature-related, with several days with maximum soil temperatures above 25 ^{o}C and some above 30 ^{o}C. There was no data to support soil water potential as a factor, but with these temperatures, there was a possibility of the top layer of soil drying out in between irrigations. This chapter investigated the effects of temperature and water potential on germination response of pre-treated seeds.

6.2: Materials and methods

For each treatment, five replicates with fifty seeds of SL2002 were stratified on two layers of filter paper in petri dishes, each with 5 mL of deionised water at 4 ^{o}C for eight weeks. The seeds were then transferred to 250 mL clear polycarbonate containers. Each container had an absorbent plastic sponge disc (45 mm diameter and 20 mm thick) topped with two layers of filter paper. The lower layer was cut and bent down so that the edges were in contact with the 25 mL of distilled water or polyethylene glycol 6000 (PEG) solutions. The upper layer was bent up to hold the seeds in place and moistened

by the lower layer which acted as a wick (Fig. 6.2.1). This allowed the seeds to be in contact with the solution but not immersed in it, to ensure adequate aeration.

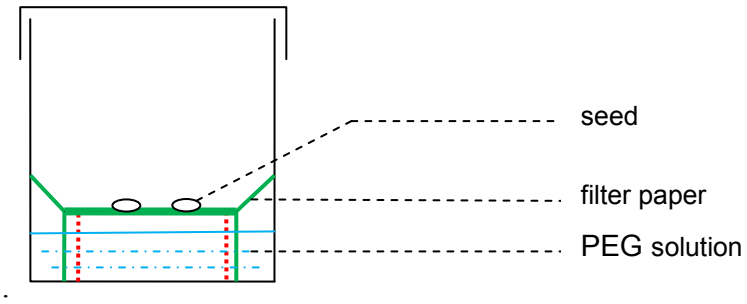


Figure 6.2.1: Diagrammatic representation of container used in the temperature/water potential experiments showing position of seeds on filter papers (green) on top of sponge disc (red) in PEG solution (blue).

All experiments in this chapter involved manipulation of seed water potential using soaking solutions of various concentrations of PEG. To establish the PEG concentrations required to obtain the required potentials at each temperature (Michel and Kaufmann, 1973; Michel, 1983), PEG solutions were measured using a vapour pressure osmometer (VPO) (Model 5100c; Wescor Inc., Logan, UT, USA) and corrected for the temperatures required using the formula:

$y = 0.0224x - 0.0564$, where y = water potential in MPa, x = VPO reading.

Experiment 1a: Temperature and water potential interactions

Germination response was measured over a range of water potentials (ψ_w) and temperatures. The containers, as described earlier, were filled with 25 mL of aqueous PEG solutions with ψ_w of 0.0, -0.1, -0.25 and -0.5 MPa. Each treatment was placed randomly along temperature zones of 10, 17, 25 & 30 ^{o}C on a Terratec thermogradient table (which has a tray of 2000 x 1000 x 25 mm and an Allen-Bradley PanelView600

programmable user-defined temperature gradient) set at 5 ^{o}C to 35 ^{o}C. The temperature zones were partitioned with polystyrene panels and covered with clear plastic covers to minimize temperature variation while allowing exposure to the laboratory lighting. The containers were left on the table for 24 hours to allow the solutions to attain the correct temperatures before the seeds were put in place.

Experiment 1b: Temperature and water potential interactions

The above method was repeated using one temperature only, 25 ^{o}C, but extending the ψ_w from 0, -0.25, -0.5, to -0.75 and -1.0 MPa. This was in response to results obtained in Experiment 1a.

Experiment 2: Time and osmotic stress and interactions

Seeds were stratified in ethephon for two weeks at 4 ^{o}C, then dried to ~30% of original dry weight (see Chapter 3) to simulate seed moisture requirement for mechanised sowing in the field. Four replicates of thirty seeds were incubated at 25 ^{o}C under osmotic stress of 0, -0.35, -0.7 and -1.5 MPa at 25 ^{o}C, then moved on Day 3, 4 or 5 to 0ψ_w for the duration of the germination test.

Germination tests, experimental design and statistical analysis

Germination tests were carried out as described earlier, with days to first germination, viability, percent final germination and mean germination time recorded. All experiments were randomised complete block designs with water potential treatments replicated as noted above. To avoid pseudo-replication (2.7 in Chapter 2), temperature treatments were not included in analyses of variance. All analyses were completed using SPSS.

6.3: Results

At 10 and 17 °C there were significant (P<0.001) water potential treatment effects on germination (Table 6.3.1). Germination percentage was reduced to 32% at 10 °C at ψ_w of -0.5MPa, and 55% at -0.25 MPa compared with 77% at ψ_w of 0 MPa. At 17 °C there was a significant reduction at –0.5 MPa but no other treatment effects. There was no significant effects on germination percentage at 25 °C, however, there was a significant treatment effect (P=0.038) at 30 °C. Viability of the seeds was not affected by either temperature or ψ_w for the duration of the experimental duration (Table 6.3.2).

There was a progressive increase in the mean germination time (MGT) at 10 °C as the ψ_w became more negative (Table 6.3.3). First day (FD) germination was on day 2 for all treatments. Figure 6.3.2 shows the effects of temperature and water potential on the germination profiles of the *E. angustifolia* seeds.

Table 6.3.1: Percentage germination of *E. angustifolia* SL2002 at five different ψ_w. Each value is a mean of 5 replicates.

	Temperature (°C)			
	10	17	25	30
MPa	%germination			
0.00	77.18	82.67	70.69	68.15
-0.10	68.55	74.96	72.22	57.81
-0.25	54.84	76.96	77.44	64.00
-0.50	31.63	57.36	74.25	52.62
LSD	*8.9*	*6.6*	*ns*	*8.7*

Table 6.3.2: Percentage viability of *E. angustifolia* SL2002 at five different ψ_w of PEG. Each value is a mean of 5 replicates. Standard error of means = 0.85±0.2.

	Temperature (°C)			
	10	17	25	30
MPa	%viability			
0.00	79.25	83.97	71.15	75.67
-0.10	78.99	77.09	73.51	73.62
-0.25	79.75	80.97	83.50	78.83
-0.50	78.34	79.94	81.96	82.29
LSD	*ns*	*ns*	*ns*	*ns*

Table 6.3.3: Mean germination time (MGT) of *E. angustifolia* SL2002 at five different ψ_w and four temperatures. Each value is a mean of 5 replicates.

	Temperature (°C)			
	10	17	25	30
MPa	MGT			
0	5.74	2.71	2.48	2.75
-0.1	5.21	2.94	2.99	3.27
-0.25	5.54	3.24	2.98	3.63
-0.5	8.39	4.11	3.74	4.33

Table 6.3.4: First day (FD) germination % of *E. angustifolia* SL2002 at five different ψ_w and four temperatures. Each value is a mean of 5 replicates.

	Temperature (°C)			
	10	17	25	30
MPa	FD%			
0	1.80	45.91	52.98	46.39
-0.1	0.82	37.55	40.22	37.14
-0.25	1.27	22.56	43.62	36.15
-0.5	0.00	4.57	36.15	12.52

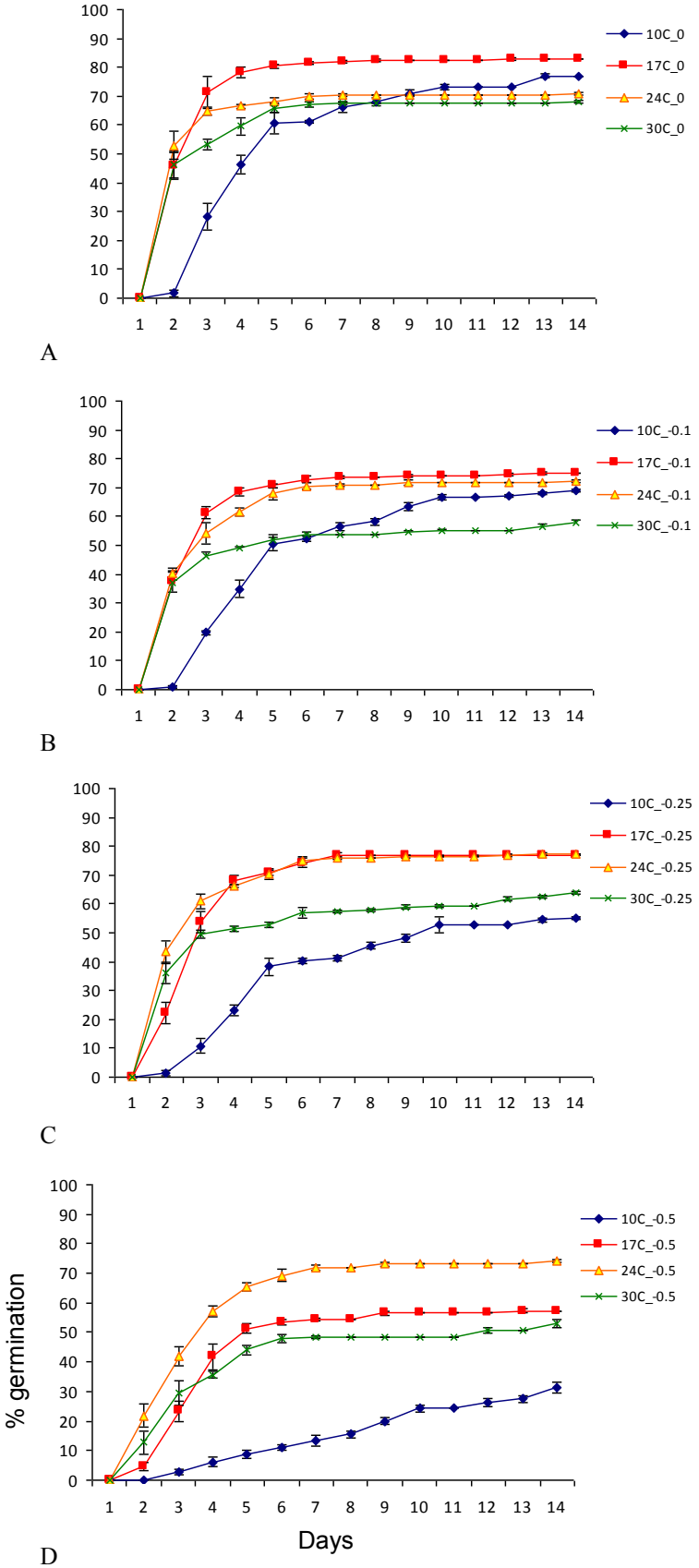


Figure 6.3.2: Germination profile of *E. angustifolia* SL2002 at osmotic potential of 0 (A),-0.1 (B), -0.25 (C) and -0.5 MPa (D) at temperatures 10, 17, 25 and 30 ^{o}C. Each data point is a mean of 5 replicates and standard error bars are shown where larger than symbol.

When the ψ_w was extended to -1.0MPa at 25 °C in Experiment 1b, both germination and viability were significantly reduced (P<0.001) to 10% and 50% respectively, compared with means of 72% and 75% for ψ_w of -0.5 MPa and lower (Fig. 6.3.3 & Table 6.3.5). There was a definite progressive decline in the FD and MGT as the ψ_w became more negative (Table 6.3.5).

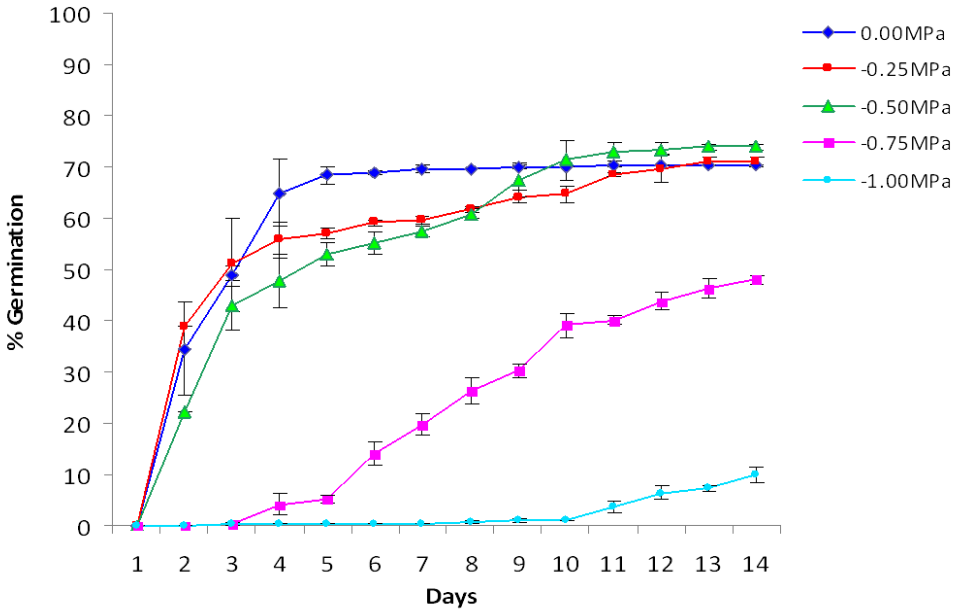


Figure 6.3.3: Cumulative germination profile of *E. angustifolia* SL2002 at 25 °C with different ψ_w of PEG 6000. Each value is a mean of 5 replicates and standard error bars are shown where larger than symbol. .

Table 6.3.5: Percentage germination and viability, first day germination, FD, (day on which the first seed germinated) and mean germination time (MGT) of *E. angustifolia* SL2002 at 25 °C with five different ψ_w of PEG 6000.

MPa	% germination	% viability	FD	MGT
0.00	70.79	72.55	2	2.99
-0.25	70.76	73.96	2	3.85
-0.50	73.04	78.91	2	4.55
-0.75	48.02	62.57	4	8.39
-1.00	9.60	49.88	8	11.64
LSD	*11.4*	*10.4*		*3.4*

Responses to osmotic stress were similar across 3, 4 and 5 days of exposure. Cumulative germination profiles (Figure 6.3.4) for Experiment 2 demonstrated no significant reduction (P=0.88) in germination percentage (mean of 80.7%) for the number of days that seeds were placed under osmotic stress. However, there was a significant (P<0.001) reduction in FD germination percentages for all exposure times as ψ_w became more negative. First day germination occurred on day one for all treatments except at –1.5 MPA when the first germinant appeared on day two. As the osmotic stress increased, FD germination decreased from a mean of 41% at 0MPa to 2% at the more negative ψ_w of -1.5MPA (Table 6.3.6). The MGT also increased significantly (P=0.005) as the seeds were stressed at more negative ψ_w (Table 6.3.7).

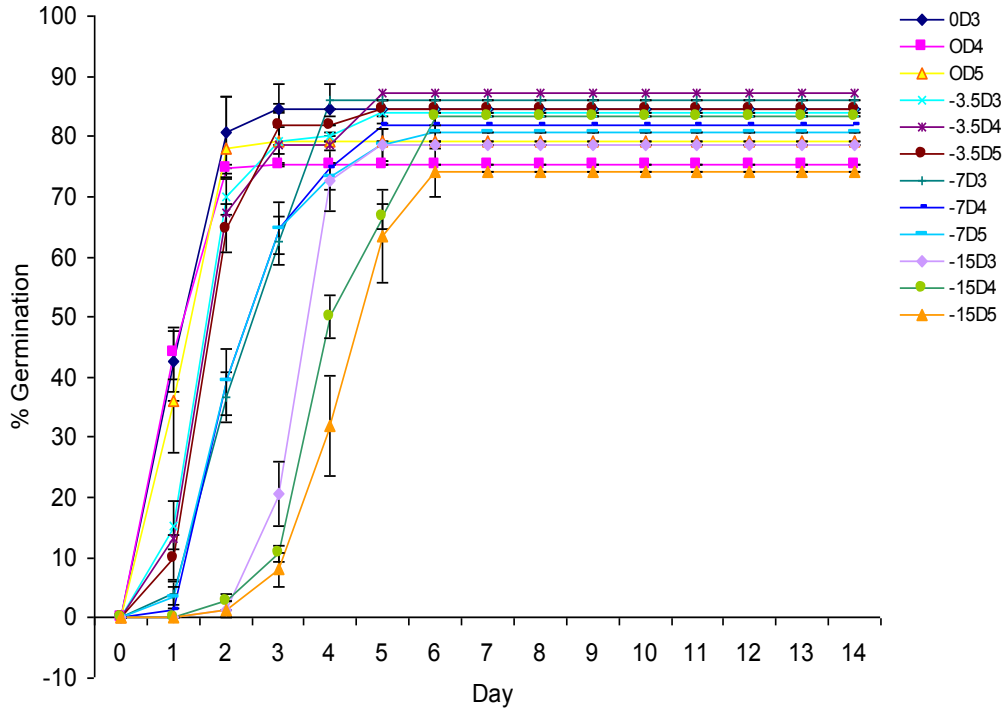


Figure 6.3.4: Cumulative germination profile of *E angustifolia* SL2002 incubated at 25 °C with ψ_w of 0, -0.35, -0.7 and -1.5 MPa, for periods of 3, 4 and 5 days (D3, D4 and D5), before removal to 0 MPa for the duration of the test period of 14 days.

Table 6.3.6: First day germination (day on which the first seed germinated) percentages of *E. angustifolia* SL2002 placed under osmotic stress for the number of days stipulated. Note: FD for all treatments was on Day 1 except for -1.5MPa which was on Day 2. LSD for comparison between means within the table=6.14.

	Time (days) under osmotic stress		
	3	4	5
MPa	FD%		
0.00	42.7	44.0	36.0
-3.50	15.3	15.3	15.3
-0.70	4.0	1.3	3.3
-1.50	1.3	2.7	1.3

Table 6.3.7: Mean germination time in days (MGT) of *E. angustifolia* SL2002 placed under osmotic stress for the number of days stipulated. LSD for comparison between means within the table =0.33.

	Time (days) under osmotic stress		
	3	4	5
MPa	MGT		
0.00	1.54	1.42	1.56
-0.35	2.09	2.27	2.18
-0.70	2.80	2.80	2.79
-1.50	3.80	4.44	4.59

6.4: Discussion

Pretreated *E. angustifolia* seedlot SL2002 seeds were able to germinate at 25 ^{o}C (T_{opt}) almost to its full potential up to an osmotic stress of -0.50 MPa. Sub-optimal temperatures down to 10 ^{o}C had no effect on the germination capacity of pretreated seeds at 0 MPa, but appeared to be associated with ψ_w in reducing germination percentages. This indicated that pretreated seeds are capable of germinating at suboptimal temperatures if not under osmotic stress. This was shown to be so in the July (winter) sowing of the field experiment (Chapter 5). The high viability percentages of

the ungerminated seeds may be an indication of induction of secondary dormancy. However, it may also be just an effect of unfavourable temperatures as those ungerminated seeds were not tested for dormancy by transferring to T_{opt}. Osmotic stress for three to five day was not significantly detrimental to the germination percentage and may even have a slight advantage (up to -0.50 MPa), as osmotic priming at -0.40 MPa was found to increase germination rate and percentage of *E. purpurea* (Pill *et al*., 1994).

The effects of suboptimal temperatures and ψ_w on metabolic activities may also have implications on the metabolic activities during the germination phase of the *E. angustifolia* seeds. Research by Dahal *et al*., (1996) on respiration and germination rates of tomato seeds at suboptimal temperatures and reduced ψ_w, found a consistency with thermal and hydrotime models. There was an exponential decline in respiration as ψ_w increased which was also observed by Ibrahim *et al*., (1983) in lettuce seeds. However, the link between respiration and secondary dormancy is unclear, although Powell *et al*., (1983) found that in secondary dormant lettuce seeds, respiration was less than 30% that of primary dormant seeds.

Above T_{opt}, germination capacity appeared to decrease but at 30 ^{o}C, the viability remained constant and there is therefore an assumption that secondary dormancy was also induced. This seemed to agree with Larsen and Erikson (2004) with *Berberis thunbergii* which required five days at 25°C to induce secondary dormancy. Thermodormancy is also a much studied subject in *Lactuca sativa* (Bewley, 1980, Kristie *et al*., 1981; Nascimento and Cantliffe, 1999) and also occurred in barley *Hordeum vulgare* L. (Leymarie *et al*., 2008, 2009) and *Avena sativa* L. (Corbineau *et al*., 1993). However, at which supra-optimal temperature dormancy was induced or

deterioration of the seeds occurred in *E. angustifolia*, was not within the scope of Experiment 1a.

Increasingly more negative ψ_w (-0.75 MPa), was detrimental to both germination capacity and viability even at the optimum temperature of 25 ^{o}C (Experiment 1b). This has implications in the field where soil moisture in the top 10 to 20 mm may dry out during periods between irrigation events, especially after sowing during the late spring/summer months.

The effects of ethephon pretreatment prevailed over ψ_w even at -1.5 MPa, although the FD was delayed, and germination percentages decreased when ψ_w became more negative. Permanent wilt potential is generally accepted as -1.5 MPa of soil water potential (Doneen and MacGillivray, 1943). Even with a five-day stress at the permanent wilt potential of -1.5 MPa, seedlings continued to survive for the duration of the germination test of fourteen days. Evans and Etherington (1990) found some weed species, associated with a drier habitat, which germinated in soil at very low potential (-1.0 MPa) and one species, *Rumex crispus*, which germinated at -1.5 MPa. This concurred with research by Doneen and MacGillivray (1943), and Manohar & Heydecker (1964). In *E. purpurea*, ethephon enhanced germination percentage of water-stress seeds ((Kochankov *et al*., 1998). It is unclear why ethephon pretreated seeds appeared to have a much better tolerance to higher ψ_w than the water pretreated seeds in Experiment 1b with higher germination % , and FD and MGT of 2 and 4.5 respectively at -1.5 MPa (Table 6.3.4&5) compared with 8 and 11.6 at -1.0 MPa (Table 6.3.3). It is possible that seeds were imbibing water from vapour or condensation in the closed environment of the container. Wuest's (2002, 2007) research suggested that water

vapour has a major role in seed imbibition, with 85% of the water absorbed by the seed in unsaturated soil being attributed to vapour alone.

The drying back of the seeds to ~30% moisture content may have recreated a matric potential in the seeds which is higher than the osmoticum allowing uptake of water. It is also possible that applying water stress for only three to five days was not long enough to have any adverse effects apart from a slower FD and MGT. This seemed to imply that the watering regime in the field may not have a huge impact but when insufficient watering occurs at temperatures below or above the optimal range, induction of secondary dormancy may take place.

6.5: Summary

The results here indicated that there is a range of temperatures from 10 ^{o}C to 30 ^{o}C at which pretreated *E. angustifolia* seeds will germinate even at osmotic stresses as low as -0.50 MPA. However, at the higher and especially the lower end of that temperature range, germination percentages were reduced and germination was delayed by more than 2.6 days with increasing osmotic stress. At the optimum temperature of 25 ^{o}C, germination was unaffected until the ψ_w increased to -0.75 MPa. The assumption that secondary dormancy was induced given the high viability percentage will need verifying with more experiments.

These results have implications with regards to the field germination of *E. angustifolia*. Particular attention to irrigation may be necessary to ensure that sown seeds are not subjected to an osmotic stress greater than -0.5 MPa for extended periods. This is

especially important when seeds are sown during warmer months when temperatures may exceed the optimal germination temperature of 25 ^{o}C.

Chapter 7: General discussion

There are several factors inhibiting the field establishment of *E. angustifolia* such as seed variability and the initial slow growth rate of seedlings with the associated agronomic problems of weed control. However, the main issue is the seed dormancy status of the species. Primary dormancy which was successfully alleviated by cold stratification *in vitro* was further complicated by the induction of secondary or conditional dormancy after sowing. The biggest problem in the establishment of *E. angustifolia* is therefore the prevention of the latter in the field.

Seed quality is an important issue where germination issues are problematic. The high cost of the seed dictates the feasibility of establishing a crop from seed versus the cost of transplanting seedlings, or not at all. The New Zealand Institute for Crop & Food Research (Douglas, 2001) found that seed sourced from Europe had 80-90% germination without a chilling treatment. Seed for this work sourced from Prairie Moon Nursery in Winona, MN, USA, also had over 90% germination. Given that there is a genetic component in dormancy (Prada *et al*., 2004; Cadman, 2006), using seeds from non-dormant seedlots may lessen the impact of problems with erratic emergence, although the induction of secondary dormancy may still be a possibility. Selective breeding of non-dormant seeds may provide a solution to eliminating the need to pre-treat the seeds.

Seed variability as defined by a seedlot's germination capacity may possibly be overcome by more precise seed cleaning methods, optimising the time of harvest and storage of seeds. Rain episodes before harvest can have a detrimental effect on

subsequent germination capacity as non-dormant seeds can undergo pre-harvest sprouting (Appendix III A). SL2002, which was used for laboratory experimentation in this work because of high primary dormancy, contained a proportion of dead seeds with dehydrated radicles (Appendix IIIB), indicating that germination had occurred either before harvest or at some stage after harvesting. The germination tests on this seedlot (pure seed fraction) would not have accounted for those seeds and would have indicated a much higher degree of dormancy than may have been the case. However, seed quality alone is not sufficient to ensure good establishment. SL2003, which achieved 74% germination *in vitro*, had less than 33% emergence (untreated) in the field experiment's spring sowings. Given that seeds may imbibe water from rainfall, then be subjected to high temperatures and then redried while still on the plant, it is questionable whether these seeds were in a primary or secondary state of dormancy.

The accepted premise that primary dormancy is present at seed dispersal may possibly be moisture-content related. *E. angustifolia* seeds obtained from an autumn 2003 harvest (whole seedheads) had a higher germination percentage (62%) when incubated immediately after removal from seedheads when compared to results (~41%) after one- and five-month dry storage. The loss of moisture during storage may have increased the level of dormancy. This is contradictory to the literature (Bewley, 1997; Leubner-Metzger, 2003) where dry after-ripening generally reduced the level of dormancy. Although outside the scope of this study, the question posed here is whether the loss of moisture increases the level of hormones such as abscisic acid (ABA) and gibberellins (GAs).

Results from experiments examining primary dormancy suggested that it may be possible to treat seeds with 10^{-3} M ethephon for a period of six hours, dried back sufficiently (~30% moisture) for the practicalities of mechanised sowing and sown on the same day. This could eliminate the need for long periods of pretreatment with associated risks of deterioration due to microbial infections. There is a possibility of slightly smaller yields with ethephon use, but the improved emergence may make up for the shortfall in yield. The length of storage (and still maintaining viability) after seeds are treated and dried back is not known for *E. angustifolia* but research on osmo-primed seeds of sunflower deteriorated faster than untreated seeds in accelerated aging (Chojnowski *et al*., 1997). In *Pinus taeda* L., storage below 10% moisture is recommended to prevent dormancy (McLemore and Barnett, 1968).

The sowing of untreated seeds of *E. angustifolia* in winter months (July for Tasmania) and allowing a natural release from dormancy under field conditions is an option. Results from this study, however, indicated that the temperature may be critical in achieving good emergence. Over 70% emergence was achieved at the Glenora site which had a larger fluctuation of temperature with more days with temperatures at 4 ^{o}C and below. However, with winter sowings, there is a longer period between sowing and plants achieving maturity, and consequently, a longer period of agronomic management. The slow initial growth of the seedlings was problematic with weed management as well as frost risk, predation and infection.

The use of pretreatments on *E. angustifolia* seeds improved germination percentage, first day germination and mean germination time in the laboratory experiments and also significantly increased field emergence in early spring sowings (September and

October). By late spring, emergence started to decline although retrieved seeds were viable indicating the reversion to conditional dormancy. This is most likely due to the increase in temperature with air and soil temperature recordings in the high 20's and some days over 30 ^{o}C.

Stratification of imbibed seeds at low temperatures promoted the loss of dormancy and improved synchrony in the germination of *E. angustifolia* but the mechanism is largely unknown. There may be several modes of action involved, such as changes in hormonal levels, interactions or sensitivity (Kucera *et al*., 2005), enzyme functions such as ß-1,3-glucanases (Leubner-Metzger, 2005), alterations in membrane structure and integrity (Simon, 1974; Hilhorst, 1998), or leaching of inhibitors (Small and Gutterman, 1991). Low temperatures may also increase sensitivity to GAs as reported by Debeaujon and Koorneef (2000) and Yamauchi *et al*., (2004), who found that an increase in the level of bioactive GAs and transcript abundance of GA-inducible genes in response to low temperatures in imbibed *Arabidopsis thaliana* seeds. GA response is linked to phytochrome involvement, although in this study of *E. angustifolia*, light was not a limiting factor. Cold stratification simulates the natural habitat conditions of *E. angustifolia* but the exact physiological and biochemical explanation is not clear. ABA involvement with dormancy induction and maintenance is well-researched and generally accepted (Karssen and Lacka, 1986; Bewley and Black, 1994; Hilhorst and Karssen, 1998; 2000).

The process of imbibition during cold stratification, inevitably results in solute leakage (Simon and Wiebe, 1974; Hendicks and Taylorson, 1976). However, publications specific to the leakage of germination inhibitors such as ABA (inhibitor hypothesis), are

limited. Webb and Wareing (1972) found ABA presence in the leachate from sycamore fruits but also found another unknown fraction which had inhibitory effects on germination essays. A seedcoat effect providing a barrier to germination was also found in *E. angustifolia* but whether it was due to inhibitors is not known (Appendix I). The effect of low temperature on imbibition may have some effects on the rate of water uptake (Leopold, 1980; Murphy and Nolan, 1982) but did not appear to impact on *E. angustifolia* (Appendix II).

Although *E. angustifolia* is a drought tolerant plant when established, the water status of the seed is critical at the germination stage. Water availability may still be an issue even when there is irrigation. Evaporation loss and drainage need to be taken into account when evaluating the water status of the seedbed (Bonachela *et al*., 2001). Properties of the soil such as infiltration, water-holding capacity, soil structure, organic matter and water potential all have an impact on the uptake of water by the seed (Cotching and Belbin, 2007). The practice of raised bed farming also may have an influence on drainage after irrigation (Clark and Edis, 2004) and decreased water availability. Soil structure and organic matter determine water filtration and retention (Franzluebbers, 2002) and therefore seed-soil contact and water availability. From the field experiment results, raised beds with adequate irrigation did not appear to pose a problem generally, although the lower November emergence at Ulverstone, with induction of secondary dormancy, may be questionably.

Results from the investigation into secondary dormancy indicated that there was an optimum temperature of 25 ^{o}C at which water potential had no perceivable effects on the germination of imbibed, pretreated seeds. However, at sub- or supra-optimal

temperatures, water potential had a detrimental effect on germination. Water potential effects at sub-optimal temperatures under field conditions were probably negligible as those temperatures mostly occurred in the colder, wetter months when the soils are generally at field capacity. Both air and soil temperature data at the experimental sites (Chapter 5) showed greater frequency of days with temperatures greater than 25 ^{o}C from mid-October onwards, which may explain the lower emergence in November. With higher temperatures, water potential effects will have greater significance as evaporation loss increases between irrigation (Myers *et al*., 1984). The relatively shallow depth (~12 mm) of the sown seed was therefore subjected to greater variations in the soil water potential (Saxton *et al*., 1986, Ritchie, 1998). From field observations, the ferrosol at the Ulverstone trial site appeared to have larger aggregates and when dry, there were larger air spaces where seed-soil contact may have been inadequate. The use of overhead irrigation may not be adequate in maintaining water potential at near 0 MPa at the initial stages of germination. Other types of irrigation such as drip irrigation for such soil types may be more consistent in supplying the water requirements to the freshly sown seeds (Bryan *et al*., 1975). Mulching may be another option to maintain water requirements. However, the elevation of temperature with plastic mulch (Li *et al*., 1999) may be a problem in the induction of secondary dormancy, and degradable polymer mulch (Halley, 2001) may not be cost effective.

7.1: Conclusions

The success of establishing a commercially viable crop of *E. angustifolia* in the field is dependent of several factors such as seed quality (dormancy status), time of sowing (temperature-dependence), water requirements (water potential) and use of seed pretreatments.

To a large extent, dormancy status is linked to genetics and therefore the use of seeds with a high non-dormant portion would be advantageous. Although no research is available regarding this in *E. angustifolia*, dormancy genes are found in other species such as *Arabidopsis thaliana* (Alonso-Blanco *et al*., 2003) and *Oryza sativa* (Gu *et al*., 2004). The use of seeds with low dormancy will allow for spring sowings without pretreatments, although the risk of induction of secondary dormancy may still be present if conditions are not suitable. Plant breeding may be a worthwhile option to obtain quality non-dormant seeds.

The sowing of seeds during winter months to take advantage of the natural climatic conditions allows seeds to undergo stratification. The advantages of this are:

- seeds do not need pretreatments with associated risks,
- there are no water potential issues as the fields are generally at capacity (irrigation will still be required at the initial stages because of soil disturbance).

The disadvantages of winter sowings are:

- preparing the beds may be problematic because of wet ground
- sites need to have a sufficient number of days with temperatures at/below 4^{o}C
- seeds are in the ground for a long period of time and therefore subject to predation and microbial attacks
- the longer time period is also problematic with weed control, frost risks, predation and infection.

Water potential effects in the field will require more experimentation. From the *in vitro* experiments, indications are that the effects can be detrimental when combined with temperature. The optimum temperature of 25 ^{o}C when water potential did not have any

effects is, naturally, not controllable in the field. Irrigation is therefore critical in the initial days following sowing.

The use of pretreatments will be necessary for spring sowings when there is a dormant portion in the seedlot. Water stratification is effective but requires eight weeks for full effect. The use of ethephon at 10^{-3} M enables the time to be reduced to two weeks but there may be some toxicity effects on plant growth. Ethephon was more effective at 4 ^{o}C but temperature below 25 ^{o}C had good germination results. The sowing of untreated seeds and spraying the bed with ethephon immediately after, is a possibility that could be explored under field conditions.

7.2: Further research

- The possibility that dormancy status is genetically linked presents further opportunities for research in that area. The ability to „switch off' the dormancy gene will present great advances in many cultivated species besides *E. angustifolia*.

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- Plant breeding from non-dormant seeds may be another possible direction in producing seeds that are free from the dormancy problems associated with the cultivation of the species.

- Time of harvesting seeds may also have an impact on the germination aspects of cultivation. Further research into the best time of harvesting may assist in

eliminating the problem of pre-harvest sprouting such that pre-germinated (therefore non-viable) seeds are not included in the next season's sowings.

- More research into the water potential issue in relation to secondary dormancy will be highly beneficial. Soil type impact on the water availability to the seed may be more significant than the watering regime.

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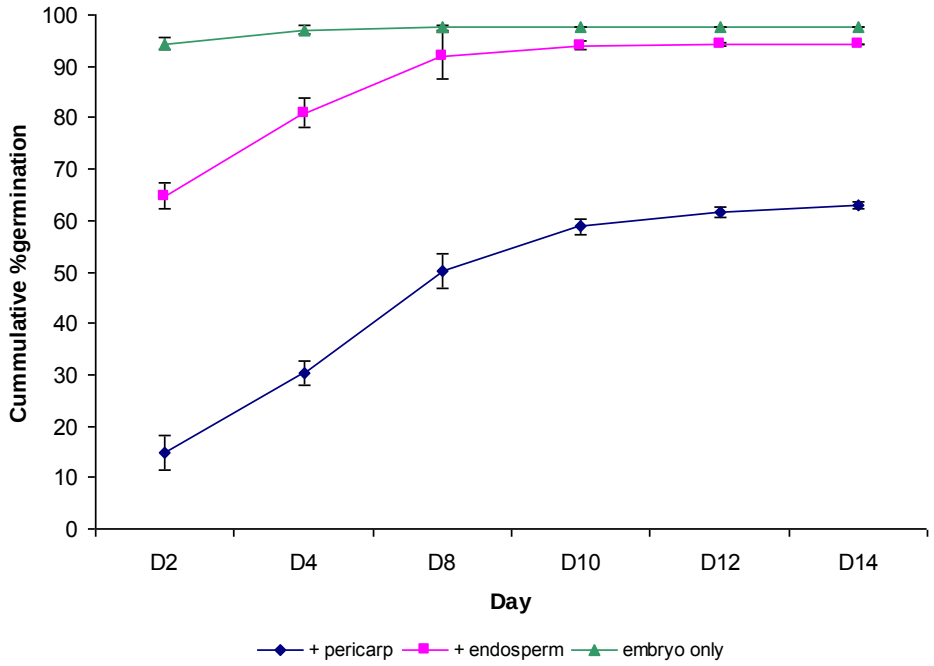
Appendix I: Seed covering effects on germination

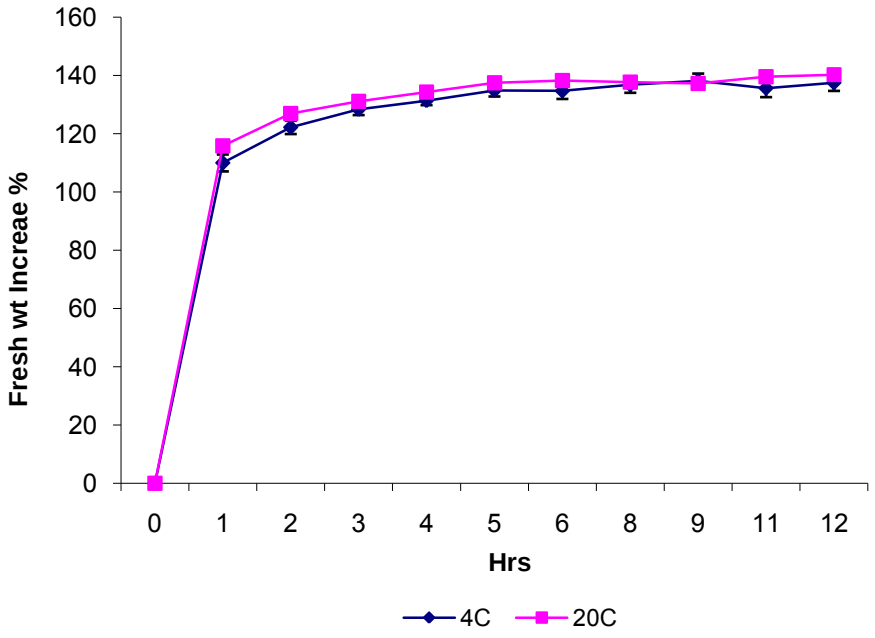


Whole seeds (achene) of *E. angustifolia* SL2003 were used for ◆. Pericarp and testa, but not the endosperm, were removed for ■, and all three layers were removed for ▲. Each point is a mean of 4 replicates with 50 seeds each. Seeds were germinated at 25 °C with 12/12 h light/dark conditions.

Embryos of *E. angustifolia* SL2003 (collected as seedheads) germinated to full capacity from day 2. Endosperm covering delayed initial germination but attained similar germination percentage as the embryos by day 10. Achenes had 30% less germination which suggested a pericarp/testa-imposed dormancy possibly by inhibitors.

This result will not have a field application as „seeds' are sown as achenes. However, it does suggest that seed pretreatments may also remove inhibitors present in the pericarp.

Appendix II: Effect of imbibition temperatures on water uptake



Seeds of *E. angustifolia* SL2002 imbibed at 4 ^{o}C and 20^{o}C. Each data point is a mean of 4 replicates with 100 seeds each.

Percentage increase in weight of achenes during imbibition of *E. angustifolia* SL2002 seeds at 4 and 20^{o}C. Seeds were weighed every hour after surface drying between two layers of paper towels. There were no appreciable differences in the rate of imbibition at these two temperatures.

Appendix III: Seed issues

A: Occurrence of pre-harvest sprouting of *E. angustifolia* SL2003 seeds in a pot trial with overhead irrigation.

B: *E. angustifolia* seedlot SL2002 showing normal seeds on the left and seeds with dehydrated radicles (seeds had germinated on seedhead before harvest or subjected to ideal germination conditions after harvest, then dried).